Review Article

Recent Advances in Proper Management of Multidrug-resistant Tuberculosis

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ABSTRACT

Widespread occurrence of multidrug-resistant tuberculosis (MDR-TB) is a serious threat to the success of TB control worldwide. Based on current evidence, the World Health Organization (WHO) has recently revised the classification of anti-TB drugs to help clinicians build an appropriate regimen for effective treatment of MDR-TB. A shorter (9 to 12 month) regimen has also shown promise in effective treatment of MDR-TB. Accurate drug susceptibility testing (DST) of *Mycobacterium tuberculosis* to anti-TB drugs is also crucial for diagnosis and management of MDR-TB. Phenotypic DST of *M. tuberculosis* by solid medium-based methods is slow, requiring 4 - 6 weeks to report results. Liquid broth-based automated Mycobacteria Growth Indicator Tube (MGIT) 960 system reporting results within 10 - 14 days have been developed and endorsed by WHO. Although performance of MGIT 960 system was excellent in early proficiency studies for first-line drugs except pyrazinamide, recent studies have shown poor performance for *M. tuberculosis* isolates with low-level resistance to rifampicin and ethambutol. Performance of MGIT 960 system for second-line drugs is also sub-optimal. Molecular DST methods rapidly detect resistance to first-line and important second-line drugs. Whole-genome sequencing is a newer alternative capable of providing rapid drug resistance profiles to inform treatment and strain information for global surveillance.

KEY WORDS: anti-TB drugs, multidrug-resistance diagnosis, recent developments, re-classification, tuberculosis

INTRODUCTION

Tuberculosis (TB) is a major infectious disease of global proportions and the widespread occurrence of drug-resistant (DR)-TB is a serious threat to global TB control success. The natural history of TB is unique. Most active TB disease cases in humans are caused by *Mycobacterium tuberculosis.* Some disease cases are also caused by *Mycobacterium africanum* (mainly in Africa) and *Mycobacterium bovis* (due to consumption of unpasteurized milk), two other species belonging to the *M. tuberculosis* complex[1]. The infection is acquired by individuals mainly by inhalation of droplet nuclei containing few bacilli expectorated by sputum smear-positive pulmonary TB patients (open TB) during close human contact[2,3]. Primary infection with *M. tuberculosis* either leads to clinically active TB disease (in ~10% of exposed individuals) or the effective immune response mounted by the host arrests multiplication of tubercle bacilli; however, complete sterilization is achieved in only a sub-set of individuals[2,3]. In the remaining subjects, infection is only contained but not eradicated, as some bacilli escape killing and persist in granulomatous lesions (latent TB infection). The latent infection may remain dormant for a long-time; however, *M. tuberculosis* retains the ability to resuscitate and cause active TB, years to decades later, often due to waning of the immune response[2,3]. Current estimates suggest that nearly 25% of the world population is latently infected with tubercle bacilli and 5 - 10% of the infected individuals will eventually develop active TB disease during their life-time[4]. The risk of reactivation of latent infection is much higher in human subjects with underlying immunodeficiencies, diabetes or co-infection with human immunodeficiency virus (HIV) [2,3]. Most active TB disease cases in low TB incidence/ high income countries occur in foreign-born individuals due to reactivation of latent infection,

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while recent infection/re-infection is also common in TB endemic countries[5-7]. Pulmonary TB accounts for > 85% of active TB cases in high TB incidence countries, while extrapulmonary TB is more common in low TB incidence countries[5-7].

METHODS

In this article, recent advances in the diagnosis and proper management of patients with DR-TB were critically reviewed. For this purpose, the latest data on the global epidemiology of TB and DR-TB were obtained from the annual TB report published in 2017 by the World Health Organization (WHO). The current literature on re-classification of anti-TB drugs, new treatment approaches and recent developments in rapid diagnosis of DR-TB were extensively researched and critically evaluated. The main findings are described below.

LITERATURE REVIEW

Global epidemiology of TB, drug-resistant TB and multidrug-resistant TB

Despite declining trends in the worldwide incidence of active TB disease by about 2% and reduction in TB deaths by nearly 3% in the last several years, the global burden of TB continues to remain high. According to the latest annual survey conducted by the WHO, there were an estimated 10.4 million active TB disease cases (including 1 million patients co-infected with HIV) in 2016[8]. Most of the estimated TB cases in 2016 occurred in the WHO regions of South-East Asia (45%), Africa (25%) and the Western Pacific (17%) while only 7%, 3% and 3% of cases occurred in the Eastern Mediterranean region, the WHO European region and the region of the Americas, respectively[8]. Nearly 56% of the 10.4 million TB cases occurred in only five (China, India, Indonesia, the Philippines and Pakistan) countries[8]. The annual number of incident TB cases varied widely among individual countries, ranging from less than 10 per 100,000 people in most high-income countries to 150 - 300 per 100,000 people in most of the 30 high TB burden countries[8]. The incidence of more than 500 cases per 100,000 people was also recorded in some countries (such as Lesotho, Mozambique, the Philippines and South Africa). An estimated 1.3 million people not infected with HIV (HIV-negative) and an additional 0.374 million HIV-coinfected individuals died from TB in 2016, making TB the ninth leading cause of death worldwide and the leading cause of death from a single infectious agent[8]. Most of the global TB deaths in recent years have been attributed to the resistance of *M. tuberculosis* strains to an increasing number of anti-TB drugs.

The increasing incidence of DR-TB, multidrug-resistant (MDR)-TB (*M. tuberculosis* resistant at least to rifampicin and isoniazid, the two most effective first-line drugs) and extensively drug-resistant (XDR)-TB (MDR-TB strains additionally resistant to a fluoroquinolone plus injectable agent, kanamycin, amikacin or capreomycin) pose a major threat to global TB control efforts[8]. In 2016, an estimated 600,000 new TB cases were resistant to rifampicin (RR-TB) and 490,000 of these RR-TB cases were additionally resistant to isoniazid (MDR-TB)[8]. Patients infected with RR-TB also require the same treatment approaches as MDR-TB. Worldwide, an estimated 4% of all new TB cases and 19% of previously treated cases had MDR-TB and nearly half (47%) of these cases occurred in only three (India, China and the Russian Federation) countries[8]. The WHO has further categorized infection with *M. tuberculosis* strains resistant only to rifampicin and isoniazid without additional resistance to other first-line drugs as uncomplicated MDR-TB. Successful treatment of uncomplicated MDR-TB is higher compared to treatment of MDR-TB resistant to additional first-line drugs[8-10]. Nearly 10% of all MDR-TB cases are now estimated to have XDR-TB[8-11]. Several countries including India, Iran, Italy and South Africa have also reported totally drug-resistant (TDR) (or extremely drug-resistant)-TB, active disease caused by *M. tuberculosis* strains resistant to all tested anti-TB drugs[12-16]. The definition of TDR-TB, however, is currently vague and not endorsed by WHO, since drug susceptibility testing (DST) results for many second-line and other drugs are poorly reproducible (ranging from 50 - 80%) and the number of drugs tested varies widely among reference mycobacteriology laboratories around the world[17]. The WHO expert committee has recently concluded that defining total drug resistance in *M. tuberculosis* is challenging and controversial and the existing category of XDR-TB already encompasses extensive drug resistance to the most active anti-TB drugs[9,17].

Treatment of fully drug-susceptible-TB is highly efficacious[18,19]. On the contrary, treatment of patients with DR-TB, particularly MDR/XDR-TB, is much more difficult due to lengthy (12 - 24 months), more expensive and more toxic drug regimens and the patients often experience clinical failure or disease relapse[10,11,16]. Worldwide, treatment success rates for drug-susceptible TB, MDR-TB and XDR-TB have been recorded as 83%, 54%, and 30%, respectively[8]. Thirty-five countries in Asia and Africa have also introduced short-course (9 - 12 months) drug regimens (known as Bangladesh regimen) for the treatment of RR-TB/MDR-TB patients, with treatment success rates of

nearly 90%[8,20,21]. Two new anti-TB drugs (bedaquiline and delamanid) have recently been approved to treat MDR-TB under defined programmatic conditions[22]. Furthermore, in an effort to improve treatment outcome, more than 80 countries have started using bedaquiline and more than 50 countries have started including delamanid in treatment regimens for MDR/XDR-TB[8].

Re-classification of anti-TB drugs for the treatment of RR-TB, MDR-TB and XDR-TB

The anti-TB drugs were previously categorized into 5 groups (Group 1 to 5) based on decreasing efficacy and increasing toxicity. Group 1 included highly efficacious, relatively less toxic and mostly bactericidal first-line (rifampicin, isoniazid (INH), ethambutol and pyrazinamide (PZA)) oral drugs suitable for combination therapy[18,19]. Streptomycin, previously used as a first-line drug, is not used routinely anymore for the treatment of fully drug-susceptible (pansusceptible) TB due to higher frequency of resistance of *M. tuberculosis* isolates to streptomycin across the world and the availability of other active drugs that can be easily incorporated in oral regimens[18,23]. Group 2 included injectable aminoglycosides (kanamycin and amikacin) and capreomycin (cyclic polypeptide)[10,19]. Group 3 included fluoroquinolones, particularly bactericidal agents such as levofloxacin (at high dose), gatifloxacin and moxifloxacin[24,25]. Group 4 included oral agents that are mainly bacteriostatic, less efficacious, expensive and more toxic than other second-line drugs and were used in therapy regimens only for treatment of MDR-TB and XDR-TB[10,18,19,26]. High dose INH and rifabutin (RBU) were also used as second-line oral agents for some patients with drug-resistant TB[10,26]. Group 5 included third-line (reinforcing agents of unproven efficacy) agents that were used only occasionally for the treatment of MDR/XDR-TB but were not recommended for routine use due to variable efficacy and serious side effects, and some of these drugs (*e.g*. thioacetazone) are contraindicated for HIV-coinfected TB patients[10,27]. These drug classifications are now considered inadequate for the proper management of MDR/XDR-TB patients. Recent estimates have shown that management of MDR-TB by conventional approaches requiring the use of multiple, highly toxic and expensive drugs for 18 - 24 months actually amplifies the antimicrobial resistance further, since successful outcome is achieved in only about 50% of treated patients[8,22,28]. The availability of two new anti-TB drugs, bedaquiline and delamanid, to treat MDR-TB has renewed hope for improved outcome of MDR-TB and to prevent development of XDR-TB[29-31]. To improve the outcome of MDR-TB treatment, WHO has now re-classified anti-TB drugs with the aim of developing a more effective (more efficacious and better tolerated) regimen for RR-TB and MDR-TB cases[32-34]. It should be emphasized here that according to the revised scheme, the currently available drugs, including bedaquiline and delamanid, are classified into four groups (Group A to Group D) specifically for the treatment of DR-TB cases, particularly MDR-TB[32-34]. Furthermore, the remaining first-line drugs (PZA, ethambutol and possibly high-dose isoniazid and rifabutin) have been relegated to a minor role as a subclass of Group D agents. The newly described anti-TB drug groups are shown in Table 1.

Group A now includes moxifloxacin, gatifloxacin or high-dose levofloxacin (fluoroquinolones with bactericidal and sterilizing activity and excellent safety profile) as the best agents for the treatment of MDR-TB. These agents are now placed ahead of injectable agents, since their use is associated with a favourable outcome[32-34]. Group B includes second-line injectable (amikacin, kanamycin and capreomycin) drugs which are bactericidal but lack sterilizing activity[32-34]. It has also been suggested that Group B may, in future, include three oral drugs; linezolid (or sutezolid or tedizolid), bedaquiline and delamanid, if they prove to be more effective and less toxic than the injectables[32-34]. Group C currently includes second-line oral drugs; linezolid, clofazimine, ethionamide/prothionamide and cycloserine/terizidone[32-34]. Linezolid is bactericidal with sterilizing action. Although linezolid at regular dose is toxic, the toxicity can be mitigated by reducing the dose[35]. Ethionamide and prothionamide have moderate bactericidal activity but also exhibit higher toxicity, while clofazimine has some sterilizing activity and good tolerability[32-34].

Group D drugs have been further dividied into three sub-groups; D1, D2 and D3. Group D1 includes PZA and other first-line drugs (ethambutol and high-dose isoniazid), provided they are likely to be effective[32-34,36]. Rifabutin may also be considered for *M. tuberculosis* isolates with specific *rpoB* mutations which confer resistance to rifampicin but not to rifabutin[37,38]. Group D2 includes two new drugs; bedaquiline and delamanid that have recently been approved to treat MDR/XDR-TB cases when no other options are available or tolerated to complete at least four active drug-regimen[22,29-31]. Both drugs are bactericidal with sterilizing activity as they target actively replicating and dormant bacilli[39-41]. Recent studies have also shown safe and effective use of bedaquiline for up to 18 months as well as concomitant

Table 1: Re-classification of anti-TB drugs for proper management of multidrug-resistant tuberculosis

Category and drug	Chemical description	Cellular process inhibited	Efficacy
Group A: Fluoroquinolones			
Levofloxacin	Fluoroquinolone	DNA replication	Bactericidal and sterilizing[a]
Gatifloxacin	8-Methoxy-fluoroquinolone	DNA replication	Bactericidal and sterilizing
Moxifloxacin	8-Methoxy-fluoroquinolone	DNA replication	Bactericidal and sterilizing
Group B: Second-line, injectable agents			
Amikacin	Aminoglycoside	Protein synthesis	Bactericidal
Kanamycin	Aminoglycoside	Protein synthesis	Bactericidal
Capreomycin	Polypeptide	Protein synthesis	Bactericidal
Streptomycin	Aminoglycoside	Protein synthesis	Bactericidal
Group C: Other core second-line agents			
Linezolid	Oxazolidinone derivative	Protein synthesis	Bactericidal and sterilizing
Clofazimine	Iminophenazine derivative	Cell membrane function	Possibily sterilizing
Ethionamide	Isonicotinic acid derivative	Mycolic acid synthesis	Weakly bactericidal
Prothionamide	Isonicotinic acid derivative	Mycolic acid synthesis	Weakly bactericidal
D-Cycloserine	Alanine analogue	Cell wall synthesis	Bacteriostatic
Terizidone	Cycloserine analogue	Cell wall synthesis	Bacteriostatic
Group D: Add-on agents			
Group D1			
Pyrazinamide	Nicotinamide derivative	Membrane energetics	Bactericidal
Ethambutol	Ethylene diimino di-1-butanol	Lipid/cell wall synthesis	Bacteriostatic
High-dose isoniazid	Nicotinic acid hydrazide	Mycolic acid synthesis	Bactericidal
Rifabutin	Rifamycin derivative	Protein synthesis	Bactericidal
Group D2			
Bedaquiline (TMC207)	Diarylquinolone	ATP synthesis	Bactericidal and sterilizing
Delamanid (OPC-67683)	Nitroimidazo-oxazole	Mycolic acid synthesis	Bactericidal and sterilizing
Group D3			
Para-amino salicylic acid	Para-amino salicylic acid	Folic acid synthesis	Bacteriostatic
Amoxycillin-clavulanate	Carbapenem with b-lactamase inhibitor	Cell wall synthesis	Bactericidal
Imipenem-clavulanate	Carbapenem with b-lactamase inhibitor	Cell wall synthesis	Bactericidal
Meropenem-clavulanate	b-lactam with b-lactamase inhibitor	Cell wall synthesis	Bactericidal
Thiacetazone[b]	Thiacetazone	Mycolic acid synthesis	Bacteriostatic

[a]Levofloxacin is bactericidal at high-dose
[b]Thiacetazone is not recommended for HIV-infected patients

use of both these agents[31,42]. These findings suggest the possibility of using these new agents for the entire duration of treatment as well as their use in patients with resistance patterns beyond MDR-TB such as pre-XDR-TB (MDR-TB strains additionally resistant either to fluoroquinolones or second-line injectable agents) and XDR-TB patients. Group D3 includes p-aminosalicylic acid, thiacetazone, amoxycillin-clavulanate, imipenem-clavulanate and meropenem-clavulanate, some of which require greater attention for toxicity[32-34]. Importantly, the meropenem/clavulanate was found to be more active than imipenem/clavulanate and is bactericidal[43-45]. Thus, meropenem/clavulanate may be used as a core drug for pre-XDR/XDR-TB cases with resistance to second-line injectables (Table 1).

According to this new drug classification proposed by WHO (Table 1), patients with RR-TB or MDR-TB should be treated with at least five effective anti-TB drugs during the intensive phase and should include PZA and four core second-line drugs, including one each from Group A and B and at least 2 drugs from Group C[32-34]. The remaining first-line drugs (ethambutol, high-dose isoniazid and/or rifabutin) are to be used only if they are likely to be beneficial based on drug resistance profile[32-34]. If a sufficient number of effective drugs are not available, an agent from Group D2 and other agents from Group D3 may be added. The new guidelines for effective treatment of RR-TB and MDR-TB also advocate that if PZA is compromised due to resistance (based on molecular analyses as phenotypic susceptibility testing is unreliable, see below) or can not be used, the regimen may be reinforced with a drug from Group C or D2, and if not possible, then from Group D3[32-34]. The total number of drugs included in the regimen should be carefully considered, keeping in mind the expected benefits and the risk of adverse reactions and non-adherence. According to WHO, recognizing and promptly managing adverse drug reactions in the treatment of MDR-TB should be considered as a priority[8,32-34]. Other important factors contributing to the success of a given anti-TB drug in the management of MDR-TB cases include easy availability of the

drug at an affordable price and reliable laboratory tests for confirming susceptibility or resistance of *M. tuberculosis* to the drug. Accordingly, high-dose isoniazid can be added to an MDR-TB regimen when a mutation in the *katG* gene is absent, however, it should not be counted as one of the four active drugs[32-34,36]. Similarly, rifabutin should be considered if susceptibility is confirmed or is suggested by a favorable *rpoB* mutation profile[37,38], but it should not be counted as one of the four active drugs.

Another important development in the treatment of MDR-TB is the introduction of the shorter 'Bangladesh regimen' of 9-months duration[20,46]. This regimen included an intensive phase of 4 months with high-dose gatifloxacin, PZA, ethambutol, clofazimine, kanamycin, prothionamide and isoniazid, followed by 5 months of continuation phase with high-dose gatifloxacin, PZA, ethambutol, clofazimine and reported treatment success rate of nearly 90%[20,46]. This regimen is much cheaper than the longer regimens that require treatment for 18 - 24 months. However, gatifloxacin, which likely played a critical role in its success, was withdrawn from the market due to the association of this drug with dysglycaemia, depriving resource-poor countries of an efficacious, effective and inexpensive drug[47]. The WHO has also recommended this shorter MDR-TB regimen in its new guidelines with moxifloxacin replacing gatifloxacin[33]. The revised shorter regimen now includes an initial phase of 4 - 6 months of treatment with PZA, kanamycin, moxifloxacin, prothionamide, clofazimine, high-dose isoniazid and ethambutol followed by 5 months of continuation phase with PZA, kanamycin, moxifloxacin and ethambutol[21,33,48]. The shorter regimen is suitable for adults and children with RR-TB and MDR-TB who have not been previously treated with second-line drugs and the *M. tuberculosis* strain has either been shown to be susceptible to fluoroquinolones and second-line injectable agents or the resistance to these agents is considered highly unlikely[33]. The WHO guidelines also recommend rapid diagnosis of drug resistance detection by molecular testing to ensure appropriate selection of patients who can truly benefit from the shorter MDR-TB regimen[33]. Rapid diagnosis will also reduce the duration of the infectious period by rapid initiation of treatment with an adequate regimen, further transmission of MDR-TB within the community and development of additional resistance leading to pre-XDR-TB and XDR-TB[21,33,48].

Drug susceptibility testing of *M. tuberculosis* to anti-TB drugs

Accurate DST of *M. tuberculosis* in clinical specimens and culture isolates to all first-line (rifampin, isoniazid, ethambutol and PZA) and important second-line (fluoroquinolones, particularly new generation fluoroquinolones, levofloxacin, gatifloxacin and moxifloxacin and injectable agents; kanamycin, amikacin or capreomycin) drugs is crucial for the diagnosis of DR-TB/MDR-TB for proper management of MDR-TB patients[49-52]. Effective treatment with sufficient number of active drugs for the appropriately required duration will also limit transmission of MDR-TB and development of XDR-TB[10,53]. Recent modelling studies have suggested that improper treatment of patients with DR-TB and MDR-TB may lead to replacement of pansusceptible TB by MDR-TB as the dominant *M. tuberculosis* phenotype across the world[54,55].

Phenotypic DST is usually considered as the most reliable laboratory approach to determine susceptibility or resistance of *M. tuberculosis* to anti-TB drugs due to good clinical correlation and quality control. Phenotypic DST of *M. tuberculosis* by solid (Lowenstein-Jensen or 7H10 agar) medium-based critical proportion method is considered as the gold standard for first-line (except PZA) and important second-line drugs. However, the method requires 4 - 6 weeks to report results[50,56,57] (Table 2). Commercial liquid culture systems and molecular assays have been developed and endorsed by WHO and Centers for Disease Control and Prevention (CDC) for more rapid detection of drug resistance in *M. tuberculosis*[50,51,58]. The liquid-broth-based semiautomated, radiometric BACTEC 460TB system accurately performed DST of *M. tuberculosis* for both first-line (including PZA) and important second-line drugs for more than two decades, reporting results within 14 days (Table 2) and was considered as an accurate and reliable alternative to the solid medium-based method[23,51,57]. The concerns for safe disposal of radioactivity, however, led to the development of fully automated culture systems such as Bactec Mycobacteria Growth Indicator Tube (MGIT) 960 system, MB/BacT system and Versa TREK system with similar turnaround time, which subsequently replaced BACTEC 460TB system in clinical microbiology laboratories[23,51,57]. Consistent results were obtained in early proficiency testing studies between BACTEC 460TB system versus MGIT 960 system or other automated systems for first-line and bactericidal second-line (fluoroquinolones and injectable aminoglycosides/cyclic peptides) drugs[51,57]. The diagnostic accuracy and reproducibility of phenotypic DST methods for less active second-line and other drugs are also inadequate as these methods have not been standardized internationally. This is reflected in the wide variability of practices among

Table 2: Common phenotypic and molecular methods used for drug susceptibility testing of *M. tuberculosis*

Phenotypic and genotypic drug susceptibility testing methods	First-line drugs tested[a]	Second-line/other drugs tested[b]	Turn-around time
Solid medium-based critical proportion			
Lowenstein-Jensen medium	INH, RIF, EMB	SM, FQs, KAN, AMI, CAP, ETH, PAS, LZD	4 - 6 weeks
7H10 agar	INH, RIF, EMB	SM, FQs, KAN, AMI, CAP, ETH, PAS, BDQ	4 - 6 weeks
Liquid medium-based critical concentrations			
Automated MGIT 960 system (m7H9 broth)	INH, RIF, EMB	SM, FQs, KAN, AMI, CAP, ETH, PAS	10 - 14 days
Automated MGIT 960 system (7H12 broth)	PZA		10 - 14 days
Hybridization-based methods			
GeneXpert MTB/RIF assay	RIF		2 hours
Real-time PCR-melting curve analysis	INH	FQs, KAN, AMI, CAP	2 hours
GenoType MTBDRplus assay	INH, RIF		1 day
GenoType MTBDRsl assay		FQs, KAN, AMI, CAP	1 day
PCR-sequencing-based methods	INH, RIF, EMB, PZA	All second-line/other drugs	1 - 2 days
Whole-genome sequencing	INH, RIF, EMB, PZA	All second-line/other drugs	1 - 2 days

[a]INH: isoniazid; RIF: rifampicin; EMB: ethambutol; PZA: pyrazinamide; SM: streptomycin; FQs: fluoroquinolones like ofloxacin, levofloxacin, gatifloxacin and moxifloxacin; KAN: kanamycin; AMI: amikacin; CAP: capreomycin; ETH: ethionamide; PAS: para-amino salicylic acid; LZD: linezolid; BDQ: bedaquiline; m7H9: modified 7H9 broth

supranational reference laboratories. Consequently, phenotypic DST for second-line and third-line drugs is not completely reliable[17,50,59,60].

Phenotypic DST by rapid liquid culture-based methods such as MGIT 960 system has been studied extensively for first-line drugs (isoniazid, rifampicin, ethambutol and PZA) with a general consensus regarding critical concentrations[38,50,57,60]. As stated above, streptomycin is now used as a second-line drug. Although the performance of MGIT 960 system has been excellent for isoniazid, recent studies have shown poor performance of this method for *M. tuberculosis* isolates for the other three (rifampicin, PZA and ethambutol) first-line drugs for different reasons[61-65] as described below.

Limitations of DST of *M. tuberculosis* by MGIT 960 system for first-line drugs

Resistance of *M. tuberculosis* to rifampicin in ~97% isolates is due to mutations in an 81-base pair (bp) rifampicin resistance determining region (RRDR) of the *rpoB* gene[66]. The resistance in the remaining 3% isolates is due to mutations in N-terminal or cluster II regions of the *rpoB* gene or in other genes[66,67]. The solid medium-based proportion method with shorter (4 weeks) turnaround time and rapid liquid culture-based MGIT 960 system fail to detect rifampicin resistance in *M. tuberculosis* strains exhibiting low-level (minimum inhibitory concentration, MIC of 0.5-2.0 μg/ml) resistance[68-71]. These low-level rifampicin-resistant strains with increased MICs below the critical concentration mostly contain mutations within RRDR, particularly at codons 511, 516, 526 and 533 or at codon 572 within cluster II region of the *rpoB* gene[63,68-71]. It should be pointed out here that I572F mutation in cluster II region of the *rpoB* gene which confers low-level resistance to rifampicin was accurately detected by the radiometric BACTEC 460TB system which has now been discontinued[69,70,72]. In one study carried out in Bangladesh and Democratic Republic of Congo, these disputed mutations accounted for >10% of all *rpoB* mutations in *M. tuberculosis* strains cultured from patients with failing therapy or experiencing relapse[70]. Furthermore, the significance of some (such as D516Y and I572F) of these disputed mutations in conferring resistance to rifampicin is indicated by gene replacement studies[73]. The patients infected with *M. tuberculosis* strains with disputed *rpoB* mutations often fail treatment or relapse, suggesting that rifampicin resistance due to disputed *rpoB* mutations is clinically and epidemiologically relevant[74-77]. These findings call for modification of the standard phenotypic DST by MGIT 960 system for greater accuracy of rifampicin resistance detection and suggest that a susceptible result should be confirmed by molecular testing when the suspicion for rifampicin resistance (such as previous history of anti-TB therapy, failing therapy, relapse or history of close contact with a patient with RR-TB and MDR-TB) is high. Molecular testing for rifampicin resistance is also important since some mutations (such as H526Y/D, S531L, *etc*.) confer cross resistance to rifabutin, while strains with other *rpoB* mutations (particularly at codon 511, 516, 533 and some mutations at codon 526) are resistant to rifampicin but remain susceptible to rifabutin[37,38,78,79]. Hence, rifabutin may be used as an alternative second-line drug in treatment regimens of some MDR-TB patients.

PZA is used for the treatment of pan-susceptible

TB as well as DR-TB and MDR/XDR-TB, as the drug is active against 'persister' bacilli that are sequestered within macrophages and are not killed by other drugs[18,80]. The drug also improves outcome in fluoroquinolone-containing regimens for the treatment of MDR-TB and new drug regimens proposed for the treatment of various forms of DR-TB also show improved outcome when combined with PZA[39-41,81,82]. Unfortunately, resistance to PZA is found frequently in MDR-TB strains, as nearly 50% of MDR-TB strains at some geographical locations are also resistant to PZA[83]. Despite these observations, PZA is still included in treatment regimens since DST for PZA is not applied in routine testing and even if applied, it often yields unreliable results[84,85]. Phenotypic DST of *M. tuberculosis* for PZA (most effective at pH 5.6) requires precise acidic conditions which prevent the growth of about 20% of the isolates[84]. Furthermore, the inoculum size also has profound effects on DST results as larger inoculum may lead to alkalization of the medium, causing false PZA resistance[85]. Nearly 90% of PZA-resistant *M. tuberculosis* isolates contain mutations in *pncA* encoding pyrazinamidase, the enzyme that converts the pro-drug PZA into its active form, pyrazinoic acid[86-88]. The *pncA* mutations are scattered across the entire length of the gene and the mutations linked with resistance have been thoroughly investigated[86-89]. Although nearly 10% of PZA-resistant *M. tuberculosis* isolates do not contain *pncA* mutation, the contribution of other genes (*rpsA* and *panD*) that have been analyzed so far appears minor, suggesting the involvement of other gene(s) [87-89]. Due to difficulties in accurate phenotypic DST for PZA, WHO is currently considering *pncA*-based methods as the recommended approach for molecular diagnosis of PZA resistance in *M. tuberculosis*[8].

Ethambutol is a slow-acting, bacteriostatic anti-TB drug and the problems associated with accurate phenotypic DST for ethambutol have been recognized for quite some time, particularly with rapid liquid culture-based methods[90,91]. Ethambutol interferes with *M. tuberculosis* growth by inhibition of one of three arabinosyltransferases (encoded by *embCAB* operon) which participate in the synthesis of arabinogalactan, a component of the mycobacterial cell wall[92]. Mutations in *embCAB* operon are the first step in the evolution of ethambutol resistance but only modestly (3 - 8 fold) increase its MIC[73,93,94]. These mutations occur most frequently (pooled sensitivity of 0.76) in *embB* gene, particularly at codons 306, 406 and 497[95]. High-level resistance, however, develops subsequently due to acquisition of additional mutations in *embCAB* operon or in other genes[96,97]. Conventional solid medium-based phenotypic DST for ethambutol is time-consuming, while DST by MGIT 960 system often reports false susceptibility of *M. tuberculosis* mainly due to mutations in ethambutol resistance conferring genes (particularly *embB*) that increase MIC close to the critical concentration of the drug[50-52,73,93,94]. The radiometric BACTEC 460TB system which has now been discontinued was much more accurate compared to MGIT 960 system for ethambutol DST, particularly for *M. tuberculosis* isolates containing *embB* mutations that confer low-level but clinically significant resistance to ethambutol[64,93,94,98]. Current evidence shows that patients infected with *embB* mutants should be considered as having EMB-resistant TB even if the isolates appear to be EMB-susceptible by phenotypic DST methods to avoid evolution of secondary mutations and selection of fully drug-resistant strains[93-97]. False susceptibility to ethambutol is not very critical for the treatment of pansusceptible TB since ethambutol is used only in the initiation phase of treatment and can even be omitted from treatment regimens if the susceptibility of *M. tuberculosis* isolate to rifampicin and isoniazid has been documented[18,80]. However, false susceptibility to ethambutol is of considerable importance for the successful treatment of RR-TB and MDR/XDR-TB as drug regimens (including shorter regimens) for these conditions should include all active first-line drugs for improved outcome[10,11,28,33,46].

Limitations of phenotypic DST methods for second-line drugs

Fluoroquinolones, particularly newer bactericidal fluoroquinolones (*e.g.* high-dose levofloxacin, moxifloxacin and gatifloxacin) and second-line injectable drugs (aminoglycosides, kanamycin and amikacin and cyclic peptides, capreomycin and viomycin) are the backbone of treatment regimens for MDR-TB, and resistance to these drugs in MDR-TB strains defines XDR-TB[8,10,32-34]. Streptomycin, another aminoglycoside which was used earlier as a first-line agent, is now used as second-line drug due to higher rates of resistance of *M. tuberculosis* strains to this agent[10,32-34]. The MGIT 960 system has also been evaluated for fluoroquinolones and injectable agents; aminoglycosides (amikacin and kanamycin) and cyclic peptides (*e.g.* capreomycin) yielding excellent agreement with the reference proportion method or with BACTEC 460TB system[50-52,59,60,99]. However, these studies have mainly been carried out with *M. tuberculosis* strains exhibiting high-level resistance to bactericidal second-line drugs. Recent studies on rifampicin-resistant and ethambutol-resistant strains indicate that *M. tuberculosis* isolates

with low-level resistance to second-line drugs may also yield discordant results more frequently with rapid liquid culture-based methods such as MGIT 960 system compared to the proportion method. The performance of MGIT 960 system for less active (mainly bacteriostatic) second-line drugs has been sub-optimal, mainly because the critical concentrations for these agents are not well-defined[50-52,99]. The problems associated with slow and/or inaccurate DST of *M. tuberculosis* by phenotypic methods have been overcome by developing molecular methods.

Molecular methods for DST of *M. tuberculosis*

Molecular methods detect genetic mutations associated with drug resistance rapidly (within 1 - 2 days) and shorten the time between MDR/XDR-TB diagnosis and appropriate treatment[100-102]. To ensure that mutations associated with drug resistance are differentiated from other mutations, specific mutations are validated by gene replacement studies[73,93,94]. Another advantage associated with this approach are the findings that different mutations confer different levels of phenotypic resistance to anti-TB drugs and some mutations are significantly associated with higher odds of patient mortality[103-106]. Based on the methodology, molecular methods are grouped into three main categories: hybridization-based assays including real-time polymerase chain reaction (PCR) assays and line probe assays, PCR-sequencing of select panel of target genes and whole-genome sequencing (WGS) of *M. tuberculosis* in clinical specimens and culture isolates[107,108] (Table 2).

Hybridization-based molecular diagnostic assays for DR-TB, MDR-TB and XDR-TB

Hybridization-based assays mainly include GeneXpert MTB/RIF assay (Xpert), reverse-hybridization-based line probe assays and various formats of DNA microarrays for detecting resistance to various combinations of first-line and/or second-line drugs. Xpert is a fully automated, cartridge-based, real-time PCR assay that detects active TB disease and resistance of *M. tuberculosis* to rifampicin[109-111]. Since nearly 85% of rifampicin-resistant *M. tuberculosis* isolates are also additionally resistant to isoniazid, the method also detects the majority of MDR-TB cases[8,23]. Another cartiridge-based, fully-automated assay that simulatenously detects resistance of *M. tuberculosis* to isoniazid, fluoroquinolones and second-line injectable agents directly in clinical specimens has recently been developed as another point-of-care test[112]. Thus, combining this test with Xpert will not only detect MDR-TB more specifically, but will also help in the diagnosis of XDR-TB. One disadvantage of the Xpert (and other hybridization-based assays) is the recent findings of silent mutations in the *rpoB* gene that lead to false rifampicin resistance by Xpert[71,113]. This has resulted in revised WHO recommendations regarding the use of Xpert. The WHO guidelines now state that Xpert may be used as the initial diagnostic test, and treatment for MDR-TB should be started if rifampicin resistance result is expected or, if unexpected, Xpert testing should be repeated on another sputum sample, particularly for settings where the prevalence of rifampicin-resistant TB is <15%[114]. Treatment, however, should be optimized following phenotypic testing or DST by another genotypic test and resolution of any discordant rifampicin susceptibility results by sequencing of the *rpoB* gene[114].

Reverse hybridization-based line probe assays that are commercially available mainly include GenoType MTBDR*plus* assay that detects resistance of *M. tuberculosis* to rifampicin and isoniazid for the diagnosis of MDR-TB and GenoType MTBDR*sl* assay that detects resistance to fluoroquinolones (levofloxacin, gatifloxacin and moxifloxacin) and injectable agents (kanamycin, amikacin and capreomycin) in MDR-TB strains for the diagnosis of XDR-TB[115,116]. Compared to other molecular assays (such as PCR-sequencing), line probe assays (and PCR-restriction fragment length polymorphism) are more suitable for the detection of developing resistance or infection with two strains, one susceptible and one drug-resistant (heteroresistance) strain as they are more easily detected by differential hybridization with wild-type and mutant probes[117,118]. However, similar to Xpert assay, line probe assays are also prone to report false resistance due to synonymous point mutations in target region[58,71,119]. Line probe assays have also been developed for *pncA* gene for the detection of resistance to pyrazinamide[120,121].

DNA microarrays have also been developed for detecting resistance to various combinations of first-line and/or second-line drugs (Table 2). A DNA microarray (GeneChip) has been developed for detection of MDR-TB and is commercially available[122]. A simplified microarray test has also been developed for detecting and identifying mutations in *rpoB, katG + inhA, embB,* and *rpsL* for reporting resistance to rifampicin, isoniazid, ethambutol and streptomycin with sensitivities (relative to phenotypic DST) of 100%, 90%, 70% and 35%, respectively. The sensitivity for MDR-TB was 89% relative to phenotypic DST[123]. However, many isolates yielded false-susceptible results due to DNA mutations that were not represented by a specific microarray probe[123]. An integrated microfluidic card with TaqMan probes

and high-resolution melting curve analysis has also been developed for detecting mutations in critical regions of *rpoB, katG + inhA, embB, rpsL + rrs + eis, gyrA + gyrB,* and *pncA* genes for detecting resistance to rifampicin, isoniazid, ethambutol, aminoglycosides (streptomycin, kanamycin and amikacin) + cyclic peptides (capreomycin and viomycin), fluoroquinolones and pyrazinamide, respectively. The test reported an accuracy of 96.1% in comparison to that of Sanger sequencing and 87% accuracy compared to phenotypic DST[124].

PCR-sequencing-based molecular diagnostic assays for DR-TB, MDR-TB and XDR-TB

PCR amplification followed by DNA sequencing (PCR-sequencing) has been used for detecting resistance to one or several first-line and second-line drugs by targeting appropriate number and regions of loci conferring resistance to different anti-TB drugs[64,72,100,125]. The sensitivity of PCR-sequencing for first-line and second-line drugs varies considerably according to the number and regions of drug resistance associated loci included for each drug. The sensitivity is also affected by the frequency of specific mutations in these loci at different geographical locations/ethnic groups of TB patients[72-74,118,126-128]. Furthermore, this approach is time consuming and technically demanding and is rapidly being replaced by WGS[101,102].

Whole-genome sequence-based assays for DR-TB, MDR-TB and XDR-TB

The problem of lower sensitivity of drug resistance detection due to limited genome coverage by hybridization-based assays and PCR-sequencing of selected panel of gene loci are overcome by WGS[101,102]. WGS characterizes both common and rare mutations predicting drug resistance, or consistency with susceptibility, for all first-line and second-line anti-TB drugs. With the advent of next generation sequencing technologies, use of WGS is increasingly being applied for routine mycobacterial species identification, detection of drug resistance and strain typing of *M. tuberculosis*[101,129-132]. The coverage of the entire genome also makes WGS a rapidly scalable method for determination of drug resistance caused by any chromosomal mutation to any first-line and second-line drug as well as newer anti-TB agents (Table 2). Recent studies have also demonstrated the applicability of WGS for tracking transmission and outbreaks of DR-TB and deciphering novel mechanisms of drug resistance[130,133,134]. Novel methods for DNA extraction from MGIT 960 cultures, optimization of library preparation, and bioinformatics pipeline have been introduced to reduce the turnaround time for obtaining WGS data[131,132]. Recent studies have also shown that WGS of *M. tuberculosis* can be performed directly from patient samples to rapidly generate antibiotic susceptibility profiles for same-day diagnosis[135,136]. England is the first country that has already started using WGS on a national scale to realize its full potential for the diagnosis of tuberculosis, detection of drug resistance, and typing of *M. tuberculosis* for epidemiological purpose[137]. However, the high cost of equipment and reagents, requirement of technical expertise and bioinformatic support make this method difficult to implement, at least at present, in resource-poor developing countries where DR-TB and MDR/XDR-TB are endemic. In this regard, it is pertinent to mention that the introduction of Xpert assay few years ago revolutionized the diagnosis of active TB and its resistance to rifampicin[109-111]. This test is currently provided at reduced cost by WHO to poor developing countries for rapid diagnosis of TB, RR-TB and MDR-TB. Similar action is urgently needed to simplify WGS data acquisition and analysis on a cost-effective basis to make it suitable for poor developing countries, if the WHO's target of 'End TB by 2035' is going to be realized.

CONCLUSION

Widespread occurrence of DR-TB and MDR-TB is mainly responsible for most of the global TB deaths. Nearly 600,000 cases of RR-TB and MDR-TB are estimated to have occurred in 2016 that resulted in the death of 240,000 patients. Accurate DST of *Mycobacterium tuberculosis* in clinical specimens and culture isolates to first-line and second-line drugs is crucial for rapid diagnosis and effective management of MDR-TB. Phenotypic DST of *M. tuberculosis* by solid medium-based proportion method is considered as the gold standard; however, the method requires 4 - 6 weeks to report results. The liquid medium-based fully automated culture systems (*e.g.* MGIT 960 system) report results within 10 - 14 days; however, their performance for *M. tuberculosis* isolates carrying specific resistance conferring mutations in target genes for some first-line drugs (*e.g.* rifampicin and ethambutol) and many second-line drugs is sub-optimal. To overcome these limitations, molecular methods have been developed for rapid (within 1 - 2 days) detection of drug resistance for all first-line and important second-line drugs. Whole-genome sequencing is a newer alternative that has the potential of providing rapid drug resistance profiles for all anti-TB drugs to inform treatment. The method additionally provides strain information for global

epidemiological surveillance. However, the cost of equipment and reagents is prohibitively high for resource-poor developing countries where DR-TB and MDR-TB are endemic. The method also requires expert technical and bioinformatic support, which makes this method difficult to implement in resource-poor and developing countries. Efforts are urgently needed to simplify WGS data acquisition and analysis on a cost-effective basis to make it suitable for poor developing countries to meet WHO's target of 'End TB by 2035' across the world.

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