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Editorial Open Access

Why Phenotypic Drug Susceptibility Testing of *Mycobacterium Tuberculosis* to First-Line Drugs is not Sufficient for Proper Management of Drug-Resistant and Multidrug-Resistant Tuberculosis?

Suhail Ahmad*

Department of Microbiology, Faculty of Medicine, Health Sciences Center, Kuwait University, Kuwait

***Corresponding author:** Suhail Ahmad, Department of Microbiology, Faculty of Medicine, Health Sciences Center, Kuwait University, Kuwait, Tel: 00965-24636503; E-mail: suhail_ah@hsc.edu.kw

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Editorial

Tuberculosis (TB) is a major infectious disease of global proportions inflicting a heavy toll on human populations. Active TB disease is caused mainly by inhalation of droplet nuclei containing few *bacilli* exhaled by sputum smear-positive pulmonary TB patients (open TB) during close human contact [1]. Primary infection with *Mycobacterium tuberculosis*, however, leads to clinically active TB disease in only ~10% of the exposed individuals. In the remaining subjects, an effective immune response mounted by the host arrests multiplication of *tubercle bacilli*, however, complete sterilization is achieved in only a sub-set of individuals [1]. 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* can also resuscitate and cause active TB, typically due to waning of the immune response [1]. Nearly one-third of the human population is latently infected with tubercle *bacilli* and 5%-10% of the infected individuals will eventually develop active TB disease during their life-time [1].

The global burden of TB and the associated morbidity and mortality are enormous. According to the latest annual survey conducted by the World Health Organization (WHO), there were an estimated 10.4 million active TB disease cases in 2016 [2]. The annual number of incident TB cases varied widely among different countries, from under 10 per 100 000 populations in most high-income countries to 150-300 per 100 000 in most of the 30 high TB burden countries [2]. More than half of all active TB cases occurred in only five (China, India, Indonesia, the Phillippines and Pakistan) countries [2]. An estimated 1.7 million individuals died from TB in 2016, making TB as the ninth leading cause of death worldwide [2]. Most of the deaths were attributed to the resistance of *M. tuberculosis* to one or more anti-TB drugs.

The anti-TB drugs are categorized as first-line (most effective), second-line (less effective, more toxic) and third-line (agents of unproven efficacy) drugs based on efficacy and tolerability [3-5]. First-line drugs (rifampicin, isoniazid, ethambutol, pyrazinamide and streptomycin) are highly efficacious, fairly affordable, relatively less toxic and mostly bactericidal oral agents suitable for combination therapy [3-5]. Streptomycin, an intramuscularly administered drug, is now mostly used as a second-line agent due to requirement for frequent patient's visits to health care facilities and higher rates of resistance among *M. tuberculosis* isolates [3-5]. Other rifamycins (rifabutin and rifapentine), though more expensive, may also be used in place of rifampicin in select patient populations [3-5].

Second-line agents are divided into three different groups; injectable aminoglycosides (kanamycin and amikacin) and cyclic polypeptides (capreomycin and viomycin), flouroquinolones (including ofloxacin, levofloxacin, moxifloxacin and gatifloxacin), and mainly bacteriostatic oral agents like ethionamide, prothionamide, D-cycloserine, terizidone, and para-amino salicylic acid [3-5]. Third-line reinforcing agents include linezolid, amoxicillin-clavulanate, meropenem-clavulanate, clofazimine and thiacetazone. The second-line and third-line agents are mainly used for the treatment of multidrug-resistant (MDR) (resistant at least to rifampicin and isoniazid, the two most effective first-line drugs) and extensively drug-resistant (XDR) (additionally resistant to a fluoroquinolone plus kanamycin/amikacin/capreomycin) TB (MDR/XDR-TB) cases due to lower efficacy and serious side effects [3-6].

The widespread occurrence of drug-resistant (DR)-TB, MDR-TB and XDR-TB strains of *M. tuberculosis* is a serious threat to global TB control efforts [5,7]. In 2016, an estimated 600 000 new TB cases were resistant to rifampicin of which 490 000 cases were resistant to both, rifampicin and isoniazid (MDR-TB) [2]. It is also estimated that nearly 10% of all MDR-TB cases now have XDR-TB which is often fatal. Several countries, including India, Iran and South Africa, have also reported totally drug-resistant (TDR)-TB strains that are apparently resistant to all tested first-line, second-line and third-line anti-TB drugs [7,8]. However, this (TDR-TB) disease entity is currently not endorsed by WHO since drug susceptibility testing (DST) for many second-line/third-line drugs are poorly reproducible (ranging from 50% to 80%), the number of drugs tested varies among reference laboratories and the existing category of XDR-TB already encompasses extensive drug resistance to most active anti-TB drugs [9].

Compared to fully drug-susceptible (pansusceptible)-TB, treatment of patients with DR-TB and MDR-TB is much more difficult due to lengthy (9-24 months), more expensive and more toxic drug regimens and the patients often experience clinical failure or disease relapse [5,7,10]. 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. Treatment of uncomplicated MDR-TB is easier and success rate for uncomplicated MDR-TB is higher compared to treatment of MDR-TB resistant to additional first-line drugs [5,7,10]. Globally, treatment success rates for TB, MDR-TB and XDR-TB have been recorded as 83%, 54%, and 30%, respectively [2]. Thirty-five countries in Asia and Africa have also introduced shorter (9-12 months) drug regimens for treatment of MDR-TB, with treatment success rates of nearly 90%. Several (>50) countries have also started using newer (bedaquiline and delamanid) drugs in treatment regimens for MDR/XDR-TB [2]. Unsuccessful treatment of MDR-TB is also a risk factor for XDR-TB,

which is very difficult to treat in most of the developing countries [5,7,10].

Accurate DST of *M. tuberculosis* in clinical specimens and culture isolates to first-line drugs is crucial for the diagnosis of DR-TB and MDR/XDR-TB for proper patient management and to limit further transmission of MDR-TB and development of XDR-TB [5,11-15]. Phenotypic DST of *M. tuberculosis* by solid (Lowenstein-Jensen) medium-based proportion method is considered as the gold standard for first-line (except pyrazinamide) and important second-line (injectable agents such as kanamycin, amikacin and capreomycin and new generation fluoroquinoloes) drugs. However, the method is very slow as it requires 4-6 weeks to report results [16-18]. 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* [15,18-20]. The liquid-broth-based semiautomated, radiometric BACTEC 460TB system accurately performed DST of *M. tuberculosis* for both, first-line (including pyrazinamide) and second-line drugs for more than two decades, reporting results within 10-14 days and was considered as an accurate and reliable alternative to the solid medium-based method [15,17,18].

The concerns for safe disposal of radioactivity have 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 [15,17,18]. These rapid systems, particularly MGIT 960 system have now replaced BACTEC 460TB system in clinical microbiology laboratories around the world. Although the performance of MGIT 960 system has been excellet for two first-line drugs, isoniazid and streptomycin, and important second-line (new generation fluoroquinolones and injectable agents) drugs, recent studies have shown poor performance of MGIT 960 system for *M. tuberculosis* isolates carrying specific resistance conferring mutations in target genes for other first-line drugs [15,18,21-23].

Resistance of *M. tuberculosis* to rifampicin in 95-97% isolates is due to mutations in an 81-base pair (bp) rifampicin resistance determining region (RRDR) of *rpoB* gene while the remaining 3-5% isolates contain mutations in N-terminal or cluster II region of the *rpoB* gene or in other genes [24]. The MGIT 960 system fails to detect rifampicin resistance in *M. tuberculosis* strains exhibiting low-level (minimum inhibitory concentration, MIC of 0.5-2.0 µg/ml) resistance [25-27]. These low-level rifampicin-resistant strains with increased MICs below the critical concentration mostly contain mutations within RRDR or at codon 572 within cluster II region of the *rpoB* gene [25-27]. Ironically, I572F mutation in cluster II region of the *rpoB* gene was accurately detected by the (now discontinued) BACTEC 460TB system [28]. The disputed (generally missed by rapid phenotypic DST methods) mutations accounted for >10% of all *rpoB* mutations in *M. tuberculosis* strains from patients with failing therapy or experiencing relapse in Bangladesh and Democratic Republic of Congo. The clinical significance of some disputed mutations is suggested by gene replacement studies [29]. Furthermore, patients infected with *M. tuberculosis* strains with disputed *rpoB* mutations often fail treatment or relapse just like patients infected with *M. tuberculosis* strains carrying canonical *rpoB* mutations [30-32]. These findings call for adaptation of the standard DST by MGIT 960 system for greater accuracy of rifampicin resistance detection. The findings also 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 rifampicin-resistant/MDR-TB) is high.

Pyrazinamide is a key drug for first-line treatment of pan-susceptible TB and second-line treatment regimens of DR-TB/MDR-TB as the drug is active against persister *bacilli* in acidic environment (within macrophages) [33]. Phenotypic DST of *M. tuberculosis* for pyrazinamide (most effective at pH 5.6) is not routinely performed because of the requirements for precise acidic conditions which prevent the growth of about 20% of the isolates [34,35]. 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 [34-36]. Nearly 90% of pyrazinamide-resistant *M. tuberculosis* isolates contain mutations in *pncA* gene [37]. Due to difficulties in accurate phenotypic pyrazinamide DST, WHO is currently considering *pncA*-based molecular diagnostics as the recommended approach for this purpose.

Ethambutol, a slow-acting first-line drug, interferes with *M. tuberculosis* growth by inhibition of one of three arabinosyltransferases (encoded by embCAB operon) that are required for the synthesis of arabinogalactan, a component of the mycobacterial cell wall [38]. Mutations in embCAB operon lead to ethambutol resistance but only modestly (3-8 fold) increase its MIC while high-level resistance develops later due to acquisition of additional mutations either in embCAB operon or in other genes [29,39,40]. Mutations in *embB* gene are more common and mostly occur at codons 306, 406 and 497 [41-43]. Phenotypic DST methods for ethambutol often report false susceptibility of *M. tuberculosis*. The radiometric BACTEC 460TB system (now discontinued) was more accurate compared to the current MGIT 960 system, particularly for isolates containing embB mutations [29,39-43]. Recent studies have shown that patients infected with embB mutants should be considered as having ethambutol-resistant TB even if the isolates appear to be ethambutol-susceptible by phenotypic DST methods to avoid evolution of secondary mutations and selection of fully drug-resistant strains [22,39,40]. False susceptibility to ethambutol is not critical for the treatment of drug-susceptible TB, however, it is detrimental for successful treatment of MDR-TB as drug regimens for this disease entity should not include ineffective first-line drugs [5,7,10,44].

Compared to the slow and/or inaccurate DST of *M. tuberculosis* by culture-based methods, molecular methods rapidly (within 1-2 days) detect genetic mutations associated with drug resistance and mainly include hybridization-based assays, PCR-sequencing of select panel of target genes and whole genome sequencing of *M. tuberculosis* in clinical specimens and culture isolates [19,20,28,45,46].

Hybridization-based assays include GeneXpert MTB/RIF assay for the diagnosis of active TB disease and its resistance to rifampicin [2,19,47]. GenoType MTBDRplus line probe assay detects resistance to first-line drugs, rifampicin and isoniazid (MDR-TB) while GenoType MTBDRsl detects resistance of MDR-TB strains for second-line drugs, fluoroquinoloes and injectable aminoglycoside/cyclic peptide drugs for detection of XDR-TB [48-50]. DNA microarrays also detect resistance to various combinations of first-line and/or second-line drugs with sensitivity of ~90% for detection of MDR/XDR-TB [51,52]. A disadvantage of these methods is the rare possibility of false resistance detection due to silent (synonymous) mutations in target regions [20,27].

PCR-sequencing has been used for detecting resistance to one or several first-line and second-line drugs and to confirm the results of

resistance detection by other methods [22,26-28,43,45]. The sensitivity of PCR-sequencing varies considerably according to the number and regions of drug resistance-associated loci included for each drug and the frequency of specific mutations in these loci at different geographical locations/ethnic groups of TB patients [42,43,48,53-55]. However, this approach is time consuming and technically demanding and is being rapidly replaced by whole genome sequencing [14,46,48].

Whole-genome sequencing (WGS) is an attractive alternative to characterise common and rare mutations in *M. tuberculosis* strains predicting resistance for all first-line, second-line, third-line and new drugs and to guide appropriate drug regimens for DR-TB and MDR/XDR-TB [46,56-58]. The method has also been used directly on patient samples for same day diagnosis [59]. Some developed countries have already started to use whole genome sequencing routinely for the diagnosis of TB, detection of drug resistance and typing of *M. tuberculosis* for epidemiological purposes [56-59]. However, the high cost of equipment and reagents and the requirement of technical expertise and bioinformatic support make this method difficult to implement, at present, in resource-poor developing countries for proper patient management where DR-TB and MDR/XDR-TB are endemic.

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