Mycobacterial Diseases
ISSN: 2161-1068

Alqahtani and Asaad, J Mycobac Dis 2014, 4:1
DOI: 10.4172/2161-1068.1000140

Research Article Open Access

Anti-Tuberculous Drugs and Susceptibility Testing Methods: Current Knowledge and Future Challenges

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Abstract

The emergence of multi-drug resistant tuberculosis (MDR-TB) outbreaks during 1990s and more recently the highly lethal strains of extensively drug-resistant TB (XDR-TB) is hampering efforts to control and manage tuberculosis (TB), and also threatening World Health Organization's target of TB elimination by 2050. The TB community has promoted a full pipeline of candidate for the new TB drugs at all phases of development. The majority of candidates are in preclinical and early clinical development. The challenge of conducting these trials, especially that they will be conducted in resource-poor locations with limited infrastructure or trials experience will be considerable. New tuberculosis drug regimens are creating new priorities for drug susceptibility testing (DST) and surveillance. To minimize turnaround time, rapid and sensitive DST will need to be prioritized, but developers of these assays will need better data about the molecular mechanisms of drug resistance. Investment to develop cost-effective and robust DST methods for peripheral laboratories or to create rapid, reliable sample transport systems to support centralized DST is questionable. Optimization of treatment regimens together with rapid diagnosis and DST for first- and second-line drugs as well as newer TB drugs, will greatly improve the prognosis and clinical outcome of TB patients.

Keywords: Tuberculous drugs; *M. tuberculosis*; Optimization; Rifampicin; Mycobacterial dormancy

Abbreviations: MDR-TB: Multi-Drug Resistant Tuberculosis; XDR-TB: Extensively Drug-Resistant TB; DST: Drug Susceptibility Testing

Introduction

Tuberculosis (TB) is still the leading cause of death from a single and curable infectious disease. In 2012, 8.6 million incident new and relapse cases of active TB disease occurred with an estimated 1.1 million (13%) of incident TB-HIV coinfected patients. The majority of TB cases worldwide were in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. India and China alone accounted for 26% and 12% of total cases, respectively [1].

Current resurgence of TB is mainly due to increasing incidence of resistance of *M. tuberculosis* strains to first-line and important second-line anti-TB drugs and the association of active TB disease with HIV co-infection or other underlying immunosuppressive conditions such as diabetes [2,3].

Data from drug resistance surveys and continuous surveillance among notified TB cases suggested that 3.6% of newly diagnosed TB cases and 20% of those previously treated for TB had multidrug-resistant TB (MDR-TB; resistance to at least rifampicin and isoniazid). An estimated 450000 people developed MDR-TB in 2012 with an estimated 17,0000 deaths from MDR-TB. Universally, a total of 94,000 TB patients eligible for MDR-TB treatment were detected in 2012, accounting for a 42% increase in the detected cases compared with 2011. Over 77,000 people with MDR-TB were started on second-line treatment in 2012 globally. On average, an estimated 9.6% of MDR-TB cases have XDR-TB [1].

There are several major problems associated with the currently available TB treatment. First, the duration and complexity of treatment result in a poor compliance to the treatment. This leads to suboptimal response (failure and relapse), the emergence of resistance, and continuous spread of the disease [4]. Second, adverse efforts to the anti-TB drugs are common and contribute to the problem of poor compliance [4,5]. Third, the increasing incidence of MDR-TB and XDR-TB causes extra serious concern. Resistant TB occurs in the presence of partially suppressive drug concentrations that enable replication of the bacteria, the formation of mutants, and overgrowth of wild-type strains by mutants (selective pressure) [6]. Fourth, the coinfection of TB and HIV is a problem by itself. The combined treatment of TB and HIV involves a high pill coast with associated compliance problems, overlapping toxicity profiles of the antiretroviral and anti-TB drugs, drug interactions between rifampicinn and the antiretroviral protease inhibitors, and the risk of immune reconstitution syndrome [7]. Fifth, prophylactic therapy of latent TB (TB infection without symptoms) with isoniazid is also associated with problems of poor compliance [8]. Attempts to shorten treatment with alternative drugs resulted in severe adverse effects [9-11].

Anti-Tuberculous Drugs Regimens

Current knowledge

The WHO Guidelines for the management of drug-resistant TB [12] have categorized available anti-TB drugs into five groups, based on known efficacy (Table 1). The WHO has developed the directly observed therapy short course (DOTS) strategy to optimize response

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Received December 12, 2013; **Accepted** January 03, 2014; **Published** January 10, 2014

Citation: Alqahtani JM, Asaad AM (2014) Anti-Tuberculous Drugs and Susceptibility Testing Methods: Current Knowledge and Future Challenges. J Mycobac Dis 4: 140. doi:10.4172/2161-1068.1000140

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Grouping	Drugs
Group 1 First-line oral agents	Isoniazid (H) Rifampicin (R) Ethambutol (E) Pyrazinamide (Z) Rifabutin (Rfb)[a]
Group 2 Injectable agents c	Kanamycin (Km) Amikacin (Am) Capreomycin (Cm) Viomycin (Vm) Streptomycin (S)
Group 3 Fluoroquinolones	Moxifloxacin (Mfx) Levofloxacin (Lfx) Ofloxacin (Ofx)
Group 4 Oral bacteriostatic second-line Agents	Ethionamide (Eto) Protionamide (Pto) Cycloserine (Cs) Terizidone (Trd) *p*-aminosalicylic acid (PAS)
Group 5 Agents with unclear role in MDR-TB treatment (not recommended by WHO)	Clofazimine (Cfz) linezolid (Lzd) Amoxicillin/clavulanate (Amx/Clv) Thioacetazone (Thz) Imipenem/cilastatin (Ipm/Cln) High-dose isoniazid (high dose H)[b] Clarithromycin (Clr)

a Rifabutin is not on the WHO List of Essential Medicines. It has been added here as it is used routinely in patients on protease inhibitors in many settings. b High-dose H is defined as 16-20 mg/kg/day.

Table 1: Alternative method of grouping antituberculosis drugs [12].

Drug	Gene	Function
Isoniazid	*kat*G	Catalase peroxidase
	*inh*A	Enoyl-acyl carrier protein reductase
	*ahp*C	Alkyl-hydroperoxide reductase
	*kas*A	Ketoacyl acyl carrier protein synthetase
Rifampicin	*rpo*B	β-subunit of the RNA polymerase
Pyrazinamide	*pnc*A	Pyrazinamidase
Streptomycin	*rps*L	Ribosomal S12 protein
	rrs	16S rRNA
Amikacin/kanamycin	*rrs*	16S rRNA
Capreomycin	*rrs*	16S rRNA
	*tly*A	rRNA methyltransferase
Fluorochinolone	*gyr*A, *gyr*B	DNA gyrase
Ethambutol	*emb*CAB	Arabinosyl transferase
Ethionamide	*gyr*B	DNA gyrase
	*inh*A	Enoyl-acyl carrier protein reductase

Table 2: Genetic basis of drug resistance in *Mycobacterium tuberculosis* [51-53].

and compliance to TB treatment. However, DOTS is labor-intensive and expensive. It causes a high burden on public health programs, especially in developing countries with limited human resources [13]. The primary backbone of tuberculosis treatment has not changed for decades. The global standard first-line TB treatment is the short-course regimen, and is used now in most high burden countries [14]. This regimen is a 6 month course of treatment denoted as 2HRZE/4HR: a 2 months intensive phase of isoniazid (H), rifampicin (R), pyrazinamide (Z), and ethambutol (E) followed by a 4 months continuation phase of H and R.

The second-line TB treatment, for patients with MDR-TB, is based only on observational studies and expert opinion [15]. These multidrug regimens of 18-24 months are toxic, expensive, and of limited effectiveness [1,16]. The inadequacy of these regimens, which has become increasingly evident as more people are diagnosed with MDR-TB, has led to efforts to find and develop new TB drug regimens that would shorten first-line treatment, avoid drug–drug interactions with antiretroviral therapy, and improve second-line treatment.

Two phase 3 trials of shorter duration first-line tuberculosis treatment have now completed patient enrolment and treatment. The OFLOTUB trial [17] replaced E with gatifloxacin in a 4 month regimen, although gatifloxacin has subsequently lost regulatory approval in many countries because of adverse effects. The REMoxTB trial [18] replaced either H or E with moxifloxacin (M) in two experimental, 4 months experimental regimens (2HRZM/2HRM and 2MRZE/2MR). Results from REMoxTB are expected in late 2013; if positive, regulatory approval will be sought in 2014 and a national launch could start as early as 2015.

Next-generation, first-line regimens are likely to include several new drugs [19]. Clinically, the most advanced regimen [20,21] in this category is known as PaMZ, a combination of the novel nitroimidazo-oxazine PA-824, moxifloxacin, and pyrazinamide. This regimen has the potential not only to shorten the duration of first-line treatment, but also to treat a proportion of patients who would previously have needed second-line treatment-i.e., patients with MDR-TB [22].

Finally, several anti-TB drugs are in clinical trials, but their optimal regimens have not yet been defined. Sutezolid (PNU-100480), an analogue of linezolid, is in phase-2a trials. More advanced are two new drugs that have been submitted for regulatory approval for treatment of MDR-TB on the basis of phase 2b data. Bacterial burden was reduced more quickly when either bedaquiline (a diarylquinoline formerly known as TMC207) [23] or delamanid (a nitro-dihydro-imidazooxazole formerly known as OPC-67683) [24] was added for 6 months, to an optimized background regimen for MDR-TB [23,24]. LL3858 is being investigated in phase I clinical trials [25,26]. A fixed-dose combination, containing LL3858; a pyrroles derivative and the standard, first-line anti-TB drugs, is also being developed [27]. However, the extent to which these drugs can shorten and simplify MDR-TB treatment will only be known after additional, multiyear phase 3 trials.

Future challenges

The rapid development of new anti-TB drugs has been hampered by several obstacles. The most important challenge in TB drug development is the difficulty to identify new compounds with activity against *M. tuberculosis*. Regimens against TB should kill both the rapidly growing mycobacteria (bactericidal activity) and the persisting mycobacteria in lesions (sterilizing activity) [28]. The molecular mechanisms responsible for mycobacterial dormancy (mycobacteria in a state of low metabolic activity and not forming colonies), persistence (drug-susceptible mycobacteria that manage to survive despite continuous exposure to TB drugs), and drug resistance are not yet fully understood [8].

Another challenge rises when evaluating new compounds, as there are currently no animal models available that predict with accuracy the required treatment duration with the newly identified compounds [29]. The guinea pig model is being explored as an alternative for the mouse model since it resembles TB pathology in humans more closely [30].

The phase of clinical testing of new anti-TB drugs is a time consuming, as the current "gold standard" to assess efficacy of anti-TB regimens in phase III clinical trials, is the relapse rate 2-years after completing the treatment. In phase-II clinical trials, the sputum culture conversion rate after 2 months of treatment is used as a surrogate marker for relapse rate, but the value of this surrogate marker is

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controversial [31,32]. Several other surrogate markers are under evaluation [32]. Large sample sizes are needed in phase III clinical trials to compare the effective standard regimen to a new regimen, even in trials that use a non inferiority design. This contributes to the length of the TB drug development process [33]. Another challenge is the scarcity of trial sites with sufficient research capacity to conduct clinical trials with large sample, especially in developing countries, where the TB burden is highest.

Drug Susceptibility Testing (DST) Methods

For decades, TB diagnosis in high-burden countries has relied almost entirely on smear microscopy, which is inexpensive but detects only half of all cases [6]. Additionally, smear microscopy does not provide any information about drug resistance, so most patients are put directly onto a standardized first-line regimen without any knowledge of drug susceptibility. However, the increasing awareness of MDR-TB has drawn greater attention to the need for DST, with the initial focus on R DST for the diagnosis of MDR-TB [34].

Phenotypic methods

The conventional phenotypic DST methods (all are indirect) include the 1% proportion method, absolute concentration, and resistance ratio, all on solid media. Because indirect methods depend on primary culture to retrieve an isolate for inoculation, DST results may take an additional 2-6 weeks. This adds up quickly to a 1-3 month delay in identification of drug resistance in patients who may suffer clinical deterioration during suboptimal therapy and who may continue to transmit drug-resistant disease to health care workers and community and family members. Thus, the alternative methods for diagnosing drug resistance, seek to provide results more rapidly, using either direct phenotypic methods for more rapid growth and detection or genotypic methods that rely on detection of mutations in known resistance-conferring regions [35,36].

The manual and automated Mycobacteria Growth Indicator Tube (MGIT) systems are commercial tests based on liquid medium originally introduced for the rapid detection of mycobacterial growth [37-39]. The MGIT system essentially cut by half the time for culture and DST results from 6-12 weeks to 3-4 weeks. One disadvantage of the MGIT is that it needs continuous, stable electricity to maintain constant incubator temperature and prevent loss of data, often requiring a back-up generator. Technical support and maintenance is essential, and some expensive reagents have shelf-lives of half a year or less upon arrival [37,38,40].

Microscopic observation broth-drug susceptibility assay (MODS) is an ‘in-house’ liquid culture method that relies on microscopic observation of serpentine cording, characteristic of *M. tuberculosis* growth. Significant advantages of MODS are its rapidity compared with solid agar culture methods, and the low cost compared with automated liquid culture systems. However, potential challenges include CO_2 supplementation and the need for meticulous technique during inoculation and plate handling to prevent cross-contamination. The requirement of an inverted microscope, in addition to biosafety concerns related to extensive handling of liquid cultures, may restrict its use to reference laboratories [40-42].

The slide DST method is similar to MODS, but it is safer and requires less equipment. However, decontamination process and ZN staining render it potentially more error proof than MODS. Quality assurance constitutes a challenge, as control strains cannot be used [43].

The microcolony method (also known as the thin-layer agar; TLA method) is performed on Middlebrook 7H11/7H10 agar for the rapid detection of mycobacterial microcolonies by conventional microscopy. Its sensitivity for detection of *M. tuberculosis* has been reported as comparable to MGIT system [44]. This method is rapid and simple, allowing simultaneous detection of TB and resistance to R and H, but more evaluation is still required. The workload as a result of repeated microscopic reading of the plates and the need for a CO_2 incubator are disadvantages [40].

The colorimetric redox indicator methods rely on the use of an oxidation reduction indicator that is added to the culture medium after *M. tuberculosis* has grown in the presence or absence of drugs. Oxygen consumption by growing cultures produces a change of color of the indicator that is easily interpreted visually. These assays do not require specific equipment and could be recommended in low-income countries, as it takes the same time to give the results as the MGIT system. Another advantage of this format is that it allows the screening of many isolates in a short period of time. Biosafety using liquid medium in microtiter plates is a concern, which can be reduced using a closed-tube format [45-47].

The Nitrate Reductase Assay (NRA) is based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite for conventional biochemical identification of *M. tuberculosis*. The main advantages of the NRA are that it is performed in classical LJ medium, with which TB laboratories are already familiar with, and there is no additional equipment is required, improving the scope for its widespread application. Results are easy to interpret by a change in color. A limitation of the NRA is that it cannot be used for the rare nitrate reductase-negative *M. tuberculosis* strains or for *M. bovis* [40].

In Mycobacteriophage-based methods, the requirement for engineered phages (e.g., Luciferase reporter phage) and the detection format (photographic film or luminometer) hampered its wide application in diagnostic clinical laboratories, and it seems more appropriate for research laboratories. Therefore, the development of a commercial format has been abandoned [48-50].

Genotypic methods

The detection of genetic mutations that are associated with resistance to certain antibiotics using molecular methods has recently become more established. They offer several advantages such as faster turnaround times and the possibility of omitting the cultures. The development of drug resistance in *M. tuberculosis* complex isolates is exclusively the result of random genetic mutations in particular genes (Table 2) [51-53].

Various molecular assays have been established, which allow for the prediction of drug resistance in clinical *M. tuberculosis* complex isolates within one working day. Generally, DNA sequencing-based approaches are considered to be the reference assays for the detection of mutations, providing the highest level of information. It can be performed by both manual and automated procedures. Automated DNA sequencing is widely used to search for mutations associated with resistance, for example, by analyzing mutations in the *rpo*B, *gyr*A, *gyr*B and *pnc*A genes [51,54-56].

Several other genotypic methods have also been developed and are in use, such as PCR-single strand conformation polymorphism (SSCP) analysis, multiplex allele-specific (MAS) PCR heteroduplex formation, hybridization assays, DNA microarrays or high-density oligonucleotide arrays, PCR-restriction fragment length polymorphism analysis (PRA)

and real-time PCR techniques. At present, a variety of real-time PCR instruments are available, together with several fluorescence formats for correlating the amount of PCR product with fluorescence signals. All real-time systems have the advantage of running the reaction as a closed system and therefore diminishing the chances of contamination. Only recently, a new real-time based PCR system was developed for the direct identification of *M. tuberculosis* complex bacteria with simultaneous detection of R resistance from specimens (Xpert® MTB/RIF, Cepheid). The system has the additional feature of being fully automated from DNA extraction to the PCR and post-PCR analysis. For this reason, this assay is of especially great value, whether or not the safety standards for culturing mycobacteria have been applied in the laboratory.

Development and field testing have led WHO to recommend line-probe assays (2008), and the Xpert MTB/RIF test (in 2010). These systems offer benefits such as reduced time to detect resistance (from effectively 106 days with conventional DST to 20 days with line-probe assay and less than 1 day with the Xpert MTB/RIF assay), [57] thus allowing for more rapid initiation of MDR tuberculosis treatment [58-60]. Liquid culture and line-probe assays can be implemented in national and regional reference laboratories, and the Xpert MTB/RIF assay (an automated, cartridge-based, real-time PCR assay) in more peripheral sites such as sub-district laboratories.

In the past few years, chip-based assays have been developed for easier and more rapid detection of resistance. These assays are commercially available, such as Combichip Mycobacteria chip or the DNA microarray (LCD array), and can detect specific mutations in the *rpo*B, *kat*G and *inh*A genes, [61,62] the TB-Biochip oligonucleotide microarray system designed to identify 29 codon substitutions and one codon deletion in *rpo*B, [63] the high-density DNA probe array for the detection of 11 distinct *rpo*B mutations, [64] or biological microchips for the detection of eight mutations in the *gyr*A gene [65]. However, as yet, these assays have not been adequately validated in different settings. Therefore, their use for clinical specimens still needs to be evaluated.

Future challenges: Despite recent market and media attention to improving MDR-TB diagnostics, it is surprising and disappointing that MDR-TB outbreaks during the 1990s [66] and more recently the highly lethal strains of XDR-TB continue to emerge worldwide [1,14,15].

It is expected that the application of molecular assays for the rapid detection of MDR-TB will increase, mainly in settings with high MDR-TB burdens. However, in high burden countries with poor infrastructure and limited resources, low-cost culture-based techniques, colorimetric assays or MODS may serve as screening tools. These methods could be promising, especially if they can be performed directly from clinical specimens and directed for DST of all second-line drugs.

The development of new DST assays address several issues: what is meant by rapid; what level of sensitivity and specificity a DST assay needs for it to be practical and feasible; and what level of complexity, containment, and cost are needed.

At present, R DST has been prioritized to diagnose MDR tuberculosis. Evidence suggests H DST should also be done: substantial numbers of patients harbor H-resistant, R-susceptible strains, and patients with such strains have reduced treatment success [67-70]. For implementation of the 4 month regimens, DST to detect susceptibility to R and fluoroquinolones will be of interest, especially in countries that already do DST for R. For the PaMZ regimen, a rapid test for moxifloxacin and pyrazinamide would probably be the first priority, because clinically significant resistance to PA-824 has not yet been shown. Development of DST for PA-824 and other new drugs will be prioritized-initially for use in surveillance-as resistance to them develops and their use becomes more widespread [34].

Another important challenge stands: New DST assays could be developed for deployment at either centralized laboratories or the more peripheral levels of the health-care system. Emerging technologies for DST are abundant and some of these technologies can be readily adapted to increase the number of mutations detected, but few are suited to use in peripheral laboratories. Therefore, investment will be needed either to develop cost-effective and robust DST methods for peripheral laboratories, or to create rapid, reliable sample transport systems to support centralized DST. One option for a peripheral laboratory test is to focus on excluding all patients who are likely to be resistant; high sensitivity becomes the goal and specificity becomes less important. A test with lower specificity can be acceptable if the prevalence of resistance is high, if an effective and safe alternative regimen (e.g., 2HRZE/4RH for PaMZ) is available, or if used as a triage test [15,33,34,59].

Another option is to continue-even with new regimens-to focus on R resistance screening as a first step. All of these theories are irrelevant without investment in the development and testing of new tuberculosis diagnostics. Those developers should be aware of what is needed in resource-limited settings and be willing to take a product all the way through field testing to commercialization [34,59,70].

Conclusion

With the emergence of MDR-TB and XDR-TB, the need for new TB drug regimens and rapid DST is intuit globally. The prospect of new TB regimens is exciting, because patients have had to rely on a single lengthy treatment option for decades. Several opportunities are available to alleviate the risks of developing resistance to these new regimens. New DST assays to detect resistance can be developed before repurposed drugs come to market, and early in the implementation of new drugs. Optimization of treatment regimens together with rapid diagnosis and DST for first- and second-line drugs as well as newer TB drugs, greatly improved the clinical outcome. Recent advances in diagnosis of MDR-TB and aggressive empirical treatment of patients with several drugs in the initial phase of treatment have further improved the prognosis of MDR-TB.

Significance of the Research

This review aimed to describe the currently available anti-tuberculous drugs regimens and susceptibility testing methods with shedding some light on the future challenges towards diagnosis and management of TB patients, especially those infected with MDR-TB and XDR-TB.

Acknowledgment

Both authors read and approved the submission of the manuscript to *the Journal Mycobacterial Diseases-Open Access.* The manuscript has not been published elsewhere and is not currently under consideration for publication by another journal.

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