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LABORATORY DIAGNOSTIC ASPECTS OF DRUG RESISTANT TUBERCULOSIS

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1. ABSTRACT

Multi-drug resistant strains of *Mycobacterium tuberculosis* (MDR-TB) are present world wide, and in many areas constitute a serious threat to the efficacy of TB control programs. The most effective strategies to limit further spread of MDR-TB are rapid detection of drug resistance followed by prompt and effective therapy. Routine laboratory diagnosis of drug resistance in TB requires a viable, pure culture of *M. tuberculosis*. Use of liquid media has decreased the turn around time for susceptibility test results however, because of the slow growth of *M. tuberculosis* these assays can still take 10 to 14 days. Alternatively, an increased understanding of the molecular basis for resistance to the antituberculosis drugs can greatly contribute to further decreasing turn around time. Based on this information, more precise and rapid molecular testing can be developed and lead to more appropriate and timely treatment regimens. In this review, we discuss methods for, and problems encountered in, performing TB drug susceptibility assays. Descriptions of routine protocols will be followed by recent developments in molecular detection of drug resistance.

2. INTRODUCTION

2.1. Antituberculosis therapy

Two drugs were introduced for TB therapy in the 1940s, streptomycin (SM) and Para-Aminosalicylic acid (PAS) (1, 2). Either SM or PAS was used alone in the treatment of TB, and initially fewer deaths were found in treated patients. However, single drug therapy resulted in emergence of drug resistant strains in a majority of the patients (3), but by combining SM and PAS, the resistance rate was reduced to 9% (4, 5). Ultimately, more effective antituberculosis drugs were introduced, and combined. Isoniazid (INH) was introduced in the early 1950s and combined with SM and PAS (6). This combination was highly effective in preventing emergence of resistance; however 18 months of treatment were required to ensure an adequate cure. Pyrazinamide (PZA), rifampin (RMP), and ethambutol (EMB) were also introduced for TB treatment and were prescribed in combinations with INH. Extensive studies to define the optimal drug combination and the minimal duration of therapy were carried out by the British Medical Research Council (7). The result was the 6 month short-course therapy consisting of 2 months treatment with

INH, RMP, and PZA, followed by 4 months treatment with INH and RMP. This protocol is still in use today, and is recommended with slight modifications (including possible addition of EMB or SM) by the American Thoracic Society, the Centers for Disease Control (CDC), the Infectious Disease Society of America, the World Health Organization (WHO), and the International Union Against Tuberculosis and Lung Disease (IUATLD) (8, 9, 10).

2.2. Drug resistance in *Mycobacterium tuberculosis*

Members of the genus *Mycobacterium* share several means of natural or intrinsic drug resistance. A hydrophobic cell envelope that serves as a permeability barrier to many compounds (11, 12, 13) surrounds these organisms. Also, drug efflux systems and drug-modifying enzymes are present in most mycobacteria, including the members of the M. tuberculosis complex (TBC) (*M. tuberculosis*, *M. africanum*, *M. canettii*, *M. microti*, *M. bovis*, *M. bovis* BCG, and *M. bovis* subspecies *caprae*) (14, 15). However, differences in natural resistance within the TBC have been observed. For example, strains of *M. bovis* and *M. bovis* BCG are resistant to PZA (16, 17, 18), and the latter is also resistant to cycloserine (19).

Acquired drug resistance, the most common type of resistance to the first-line drugs (20), can emerge against any antituberculosis agent during chemotherapy. In contrast, primary drug resistance is defined as the presence of drug-resistant organisms in a previously untreated person, presumably because that person has been infected with a strain that had acquired drug resistance in another host (21). Recently, these classifications have been revised because of difficulties in verifying the exact time when drug resistance developed. In addition, DNA fingerprinting studies have shown that resistance acquired during the course of therapy can also be the result of exogenous re- or super-infection with a second strain that is drug-resistant (22). Thus, the WHO recommends use of the term "drug resistance among new cases" instead of primary resistance, and "drug resistance among previously treated patients" instead of acquired resistance (23).

Cross-resistance can occur between drugs that are chemically related and/or have the same or similar target within the mycobacterial cell. For example, approximately 70 to 90% of RMP-resistant strains are also resistant to rifabutin (24, 25), and some strains of *M. tuberculosis* with low-level resistance to INH are cross-resistant to ethionamide (26). However, no cross-resistance has been seen between INH and PZA, even though both drugs are analogs of nicotinamide.

In the U.S., susceptibility testing of a patient's initial isolate of the *M. tuberculosis* complex is recommended by the National Committee for Clinical Laboratory Standards (NCCLS), to ensure the most effective treatment for the individual (27, 28). Performance of susceptibility testing in larger hospitals or public health reference laboratories provides accuracy of testing, and enhances the communication of resistance trends to TB control surveillance databases. It is also recommended that repeat susceptibility testing should be performed if specimens from a patient remain positive following 3 months of treatment, or if there is clinical evidence of failure to respond to therapy (10).

2.3. Development of drug resistance in *M. tuberculosis*

Drug resistance in *M. tuberculosis* occurs when resistant mutants, naturally occurring in the mycobacterial population, are selected by inadequate or interrupted treatment with antituberculosis agents (29, 30, 31). Genetic studies have confirmed that mutations in genes encoding drug targets or drug activating enzymes are responsible for resistance, and point mutations and / or deletions have been found for all first-line drugs (reviewed in 26, 32, 33, 34). Thus, strains of MDR TB obtained from worldwide sources are not the result of a single genetic event, but of successive events in different loci.

Mutants resistant to a given drug occur approximately once in every $10^7$to 10^{10} cells, suggesting that the occurrence of mutants resistant to two drugs simultaneously would in theory require a population of approximately 10^{16} mycobacterial cells. Consistent with this theory, it was found that mono-therapy led to selection of drug-resistant populations more frequently in cases of cavitary disease, in which lesions contain abundant tubercle bacilli (up to 10^9 / lesion), than in cases with non-cavitary lesions, which contain relatively few cells (about 10^{3-4} / lesion) (35). However, other factors can influence the emergence of drug resistance. Although the tubercle bacillus replicates actively in vivo, it has a long generation time and a tendency to shift its metabolism toward a dormant state. Such variations in metabolic activity make this organism a difficult therapeutic target. In addition, penetration of antibiotics to various body sites can vary significantly, resulting in some lesions with suboptimal concentrations of drugs and thus a greater opportunity for selection of drug resistant mutants. The individual patient's influence on the development of drug resistance should also be considered. It has been found that the tuberculosis patients most likely to produce drug resistant mutants are those who are experiencing increases in the bacterial population, along with compromised drug penetration due to underlying host conditions (reviewed in 36). Further study is needed to fully understand and counteract the most significant factors influencing the emergence of drug resistance in *M. tuberculosis*.

2.4. Critical drug concentrations and their relationship to determination of resistance

Early investigators in the field of laboratory diagnosis of drug resistance faced two significant challenges. First, drug concentrations (critical concentrations) necessary to eliminate susceptible (wild type) strains of *M. tuberculosis* needed to be determined. Secondly, it was unknown what proportion of a population of *M. tuberculosis* would need to be resistant to a drug in order for that strain to be interpreted as resistant, thus permitting accurate prediction of therapeutic failure.

With regard to the first challenge of determining critical concentrations, the concept of using drug concentrations achievable in blood or serum (37) was

considered; however, it was found that the levels in blood were not always the same as those achieved in target tissues. Fortuitously, it was observed that drug-susceptible strains of *M. tuberculosis* that have not been exposed to antituberculosis drugs (wild type strains) do not exhibit much variation in the minimum inhibitory concentrations (MICs) to those drugs (37). Thus, it was ultimately determined that the critical concentration should be the concentration that inhibits the growth of wild type strains without appreciably affecting the growth of resistant cells (5, 38, 39, 40). It was also found that the critical concentration in the different susceptibility assays varies slightly dependent upon the media used (40). (Table 1 lists the MICs of drug susceptible strains of *M. tuberculosis*, and the critical concentrations for three commonly used media.)

Since drug resistance develops by natural selection of mutants preexisting in the bacterial population, both drug-resistant and drug-susceptible cells will be present during the early stages of resistance development. Thus, clinically significant resistance to all antituberculosis drugs was defined as in vitro growth, in the presence of the critical concentration of the drug, that is equal to or greater than 1% of the growth in the absence of the drug (28, 40, 41). This policy is justifiable, since for a wild type strain of *M. tuberculosis*, the proportion of potential resistant mutants (based on a mutation rate of 10^{-7}) would theoretically include only 0.00001 % of the entire population. Thus, the presence of 1% resistant cells represents a substantial increase in resistance.

3. CURRENT METHODS FOR SUSCEPTIBILITY TESTING

Of all the assays performed in mycobacteriology laboratories, drug susceptibility testing has been the most difficult to standardize (41). There are a variety of reasons for the lack of standardization, including differences in the drugs and their effects on the organism, and differences in test methods and media used. In 1987, Laszlo and colleagues (42) found that, in Canadian laboratories, the reproducibility of drug susceptibility testing results was higher for INH and RMP than for SM and EMB. More recently, the WHO and the IUATLD continued these studies on the quality assurance program for susceptibility testing of *M. tuberculosis* in their supranational laboratory network (43, 44). The results confirmed the findings from the Canadian study, in that drug susceptibility testing procedures for the testing of INH and RMP, the two antituberculosis drugs that define multi-drug resistant (MDR) TB, were highly reliable within their network. However, it was found that intra-laboratory reproducibility for susceptibility testing for the other two first-line drugs, SM and EMB, has improved, but was still lower than that for INH and RMP, as will be discussed in more detail in Section 3.2.

In addition to the issue of interlaboratory reproducibility for certain drugs, a significant problem in TB susceptibility testing is the acquisition of a standardized inoculum. Because it is important to determine the proportion of resistant cells in a population at the time of susceptibility testing, the suspension that will be used to inoculate the drug-free and drug-containing media must be standardized. Obtaining a standardized inoculum can be a problem in susceptibility assays for many organisms, but for *M. tuberculosis*, it is significantly more difficult yet, due to the extensive intercellular adhesiveness (cording and clumping). For example, Canetti and colleagues (45) reported on the range of CFU counts obtained from suspensions of tubercle bacilli defined as "1 mg wet weight /ml". A group of 344 strains were tested once each, and a single strain (H37Rv) was tested 55 times. The numbers of CFU / mg / ml for both the 344 strains and H37Rv ranged from less than 10^6 to greater than 10^8 (with 62% between 5 x 10^6 and 5 x 10^7). Because of this inevitable variability, special care must be taken to obtain well-dispersed suspensions of TB bacilli (39, 40).

Susceptibility determinations may be performed as a direct test (inoculation with the decontaminated sputum specimen) or as an indirect test (inoculation with growth from a primary culture). Although there are differences of opinion on which is the best approach, the indirect test is considered superior in many laboratories since the inoculum size is uniform and the bacteria are metabolically active. In addition, inoculum from direct testing is more apt to contain contaminating normal flora. However, direct testing will usually provide more rapid results, and may more precisely represent the proportions of susceptible and resistant bacteria present in the patient.

3.1. Radiometric method (BACTEC) for first-line drugs

In 1975, Cummings and colleagues (46) reported the detection of *M. tuberculosis* metabolic end-products using a ^{14}C-labeled substrate. Based on this result, a rapid method for susceptibility testing was developed (47, 48, 49, 50). In the BACTEC system (BD Diagnostic Systems, Sparks, MD) tubercle bacilli metabolize the ^{14}C-labeled palmitic acid present in Middlebrook 7H12 liquid medium, resulting in the release of quantifiable $^{14}CO_2$. The BACTEC TB 460 instrument detects the $^{14}CO_2$ that is released (expressed as the Growth Index or GI) and then automatically replaces the air in the headspace with 5 to 10% unlabeled CO_2, thereby maintaining the recommended CO_2 atmosphere. The rate and amount of $^{14}CO_2$ produced are directly proportional to the rate and amount of growth, and procedures have been developed to use this system for testing the metabolism of the organism in the presence of all first-line drugs (SM, INH, RMP, EMB, PZA). In many laboratories, the rapid availability of results using the radiometric procedure (1 week, versus at least 3 - 4 weeks when using solid media) has taken precedence over cost considerations and the necessity for disposal of radioisotope, since the faster turnaround time often results in better patient management (51, 52).

3.2. Concentrations of first-line drugs used in the radiometric assay.

RMP is considered the most important antituberculosis drug. In 1986, the British Medical Research Council (53) found that the response of the few patients with initial resistance to RMP was poor, in contrast to the higher success rate in patients with initial resistance

Table 1. Minimal inhibitory concentrations (MICs) of drug-susceptible strains of *Mycobacterium tuberculosis* and critical concentrations for radiometric, agar proportion and Loewenstein-Jensen medium methods.

Antimicrobial agent	MICs[1]	BACTEC 12B[2]	7H10[3]	LJ[4]
Isoniazid (INH)	0.05 – 0.2	0.1 and 0.4	0.2 and 1	0.2 (0.1 and 1)
Rifampin (RMP)	0.25 – 0.5	2	1	40 (20)
Pyrazinamide (PZA)	20 – 50	100[5]	NR[6]	400 (100)
Ethambutol (EMB)	1 – 5	2.5 and 7.5	5 and 10	3 (2)
Streptomycin (SM)[7]	2 – 8	2 and 6	2 and 10	4
Amikacin	1	1	4	NR
Capreomycin	1 – 50	1.25	10	40 (20)
Cycloserine	5 – 20	NR	NR	40 (30)
Ethionamide	0.6 – 2.5	1.25	5	40 (20 and 30)
Kanamycin	5	5	5	30 (20)
Ofloxacin	0.5 – 2.5	2	2	2
PAS	1	4	2	0.5 (0.25 and 1)
Rifabutin	0.06 – 8	0.5	0.5 and 1	NR

[1] MIC values (μg/ml) taken from references 28 and 54. [2] Values (μg/ml) taken from references 28 and 100 for use in the radiometric assay in liquid medium, using BACTEC 460 instrument. [3] Values (μg/ml) taken from reference 28 for use in the agar proportion method using Middlebrook 7H10 agar. [4] Values (μg/ml) taken from references 40, 54 and 99 for use with Loewenstein-Jensen medium. [5] PZA tested at pH 6 in BACTEC 12B medium. [6] No recommendation given. [7] Dihydrostreptomycin sulfate, considered to be a second-line drug in most recent U.S. documents (10, 27).

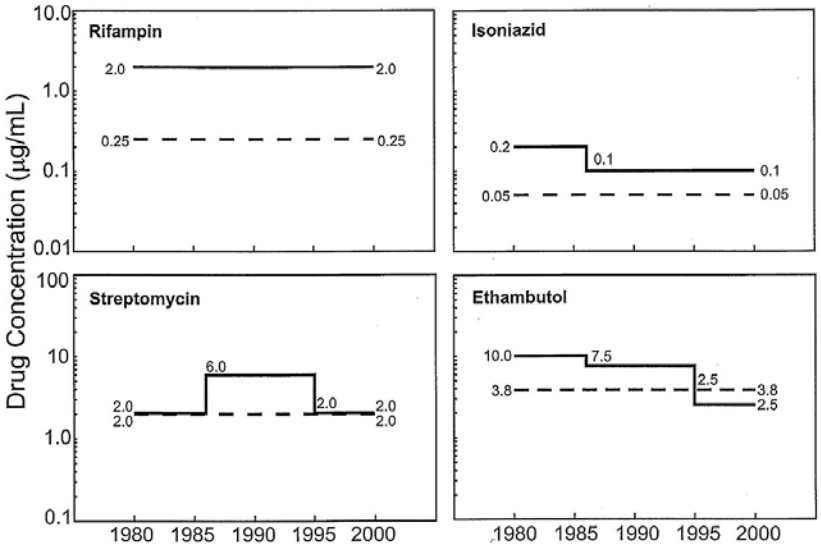

Figure 1. Continual adjustments to the recommended drug concentrations for use in the radiometric bactec 460 TB. Each graph represents the recommended critical concentration for each drug (solid line) and the MIC value (dotted line) for susceptible M. tuberculosis. The BACTEC MIC values were taken from reference 54. BACTEC concentrations and dates of changes were provided by BD Diagnostic Systems, Sparks, MD.

to INH and / or SM. Because recognition of RMP resistance is so vital for positive patient outcome, it is important to always test for susceptibility to RMP. Fortunately, the MIC of susceptible strains to RMP is below 0.5 μg / ml (0.25 - 0.5 μg / ml (28, 54)), and RMP has always been tested in the BACTEC system at 2 μg / ml (Table 1 and Figure 1). Usually, these differences result in a clear distinction in growth kinetics between RMP-susceptible and -resistant strains in the presence of 2 μg / ml RMP, the concentration that is reported.

The situation with INH is more complicated since it is often more informative for patient management to test two concentrations: 0.1 μg / ml and 0.4 μg / ml. Through the testing of the two concentrations of INH, there are three possible combinations of results and subsequent actions. If the isolate is susceptible at both concentrations, treatment with INH should be continued. If the isolate is resistant at 0.1 and susceptible at 0.4, opinions differ as to whether to continue treatment with the drug. Several studies have provided evidence that patients with primary (but not acquired) INH resistance often contain a proportion of susceptible organisms (perhaps in a state of suspended growth); and thus, it may be beneficial to continue treatment with INH (7, 55). However, if the isolate is resistant at both concentrations, INH should be replaced,

Table 2. BACTEC results: Number of isolates [1] resistant to streptomycin (SM) and ethambutol (EMB) if interpretation made at low or high drug concentration

Year	Number of isolates tested	Number interpreted as resistant at each concentration			
		Low SM (2.0)	High SM (6.0)	Low EMB (2.5)	High EMB (7.5)
1996	554	68	40	102	31
1997	660	60	40	183	27
1998	547	57	22	152	23
1999	479	40	28	114	12
2000	480	62	31	92	15
2001	375	65	46	81	17
2002	403	47	27	61	11
TOTAL	3,498	399	234	785	136
Percentage of total	100%	11.4 %	6.7 %	22.4 %	3.9 %

[1] Isolates tested at the Wadsworth Center, Clinical Mycobacteriology Laboratory

most optimally with two other drugs, especially in acquired resistance following re-treatment or persisting disease (Denis A Mitchison, personal communication). For the vast majority of hospital-based microbiology laboratories, it may be sufficient to test INH at the manufacturer's recommended concentration (0.1µg / ml). However, in settings where drug resistant strains are seen more often (e.g., public health laboratories or specialized regional laboratories), both concentrations can be tested, with the results of testing at 0.4 µg / ml reported only if resistance is seen at 0.1 µg / ml.

Standardization of the critical concentrations of SM and EMB to be used in the radiometric procedure has not been achieved without controversy (54). It is noteworthy that the recommended concentrations for SM and EMB underwent adjustments over time (56). In 1986, Hawkins (57) stressed the need for further changes with these drugs. Figure 1 depicts the documented MIC values for susceptible strains of *M. tuberculosis* (dotted line) (54), as compared to the drug concentrations that have been recommended by the manufacturer for use in the radiometric assay (solid line). The most recent changes to the critical concentrations of SM and EMB occurred in 1995, in reference to data generated by Woodley at the CDC in Atlanta (58). In that 1986 study, it was suggested that, to improve the level of accuracy of SM and EMB testing, SM should be tested at 2 µg / ml and EMB at 2.5 µg / ml (rather than the previously recommended 6.0 and 7.5 µg / ml for SM and EMB, respectively). Unfortunately, these recommended concentrations are now at or below the MIC for susceptible strains as determined by Heifets (SM, 2 µg / ml; and EMB, 3.8 µg / ml (54, 59, 60, 61)). These modifications have resulted in questions, particularly about whether the use of 2.5 µg / ml can accurately determine resistance to EMB (62). As a follow-up on these concerns, 7 years of accumulated data (from 1996 through 2002) from the New York State public health laboratory (Table 2) consistently demonstrate that, if the lower (recommended) concentration results had been reported, resistance levels would be close to 2-fold higher for SM, and 6-fold higher for EMB. Similar results were seen in data from the Florida State Tuberculosis Laboratory: SM resistance levels would have been 3-fold higher, and EMB would have been 6-fold higher if the lower concentration results had been reported (data not shown; results kindly provided by Yvonne Hale). Altogether, these results suggest that growth in the presence of the lower concentration is not indicative of true resistance to either of these two drugs. This hypothesis is strengthened, at least for EMB, by molecular data demonstrating that only strains resistant at both 2.5 and 7.5 µg / ml exhibited mutations in the drug target gene, *embB*, while susceptible strains and strains resistant at only 2.5 µg / ml had no mutations in this region (63). Other genetic loci have recently been shown to be involved in EMB resistance (64); however, all of the resistant strains included in that study were resistant at the higher concentration, 7.5 µg / ml, with MICs ranging from 10 to >50 µg / ml.

3.3. Methods for PZA testing

PZA is one of the most important drugs in the chemotherapy of tuberculosis, because it has allowed the shortening of antituberculosis therapy to 6 months (65, 66, 67). However, testing the susceptibility of *M. tuberculosis* to PZA was initially found to be problematic, since this drug is active only at an acid pH (68), and a significant number of isolates of *M. tuberculosis* would not grow at a pH of 5.5 (69, 70, 71). Subsequently, a more simplified radiometric assay using pH 6.0 was described (72, 73). This method resulted in well-defined differences between PZA-susceptible and PZA-resistant strains, and is the basis for the current recommended assay. However, there are still caveats to be considered in ensuring that the low pH is maintained in the vials. Either a large inoculum size (> 10^7) or the presence of bovine serum albumin (BSA) can reduce the activity of PZA, by neutralizing the acidic pH in the former case, or by binding the activated drug in the latter case (74, 75, 76).

Miller and colleagues (77) encountered another difficulty when they found that the radiometric susceptibility test results from approximately 3.5% of the clinical isolates could not be interpreted, because of inadequate growth. This problem may be due to poor growth of some isolates of *M. tuberculosis* at pH 6.0, or to inhibition of growth by the polyoxyethelene stearate (POES) supplement. It is possible to directly test for growth inhibition in each test by including control vials at pH 6.8 and 6.0, and test vials at pH 6.0 with PZA, all with and without POES. This protocol determines growth

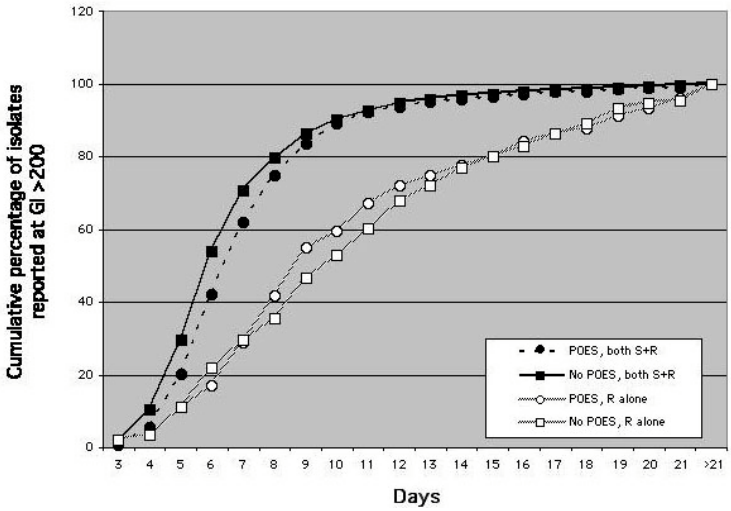

Figure 2. Days to report PZA results for 1799 *Mycobacterium tuberculosis* complex isolates (1653 Susceptible [S] and 146 Resistant [R]) in BACTEC 12B, pH 6.0,with and without POES. BACTEC vials were supplemented with either POES or sterile water and read daily on the instrument until the GI was greater than 200. These unpublished results were obtained from 1799 isolates tested at the Wadsworth Center Clinical Mycobacteriology Laboratory (1994-2001).

kinetics under various conditions, recognizing when acidic pH or POES might be inhibiting the growth of a particular strain. A comparison of the results obtained for 1,799 isolates of the *M. tuberculosis* complex (Figure 2, black lines) demonstrates that there is no significant difference in the time required to reach a GI of 200 at pH 6.0 (when the results can be interpreted and reported) in the presence or in the absence of POES. However, when 146 PZA-resistant isolates were evaluated separately (Figure 2, grey lines), POES appears to provide a slight growth advantage. These results are consistent with a previous report demonstrating that POES slightly enhances the growth of PZA-resistant strains at pH 6.0 (72). However, since PZA-resistant strains are not often encountered, the extra expense of using POES could be eliminated for routine testing, as also suggested by others (76). For example, if the 1,799 isolates had been screened without POES, the assay would have had to be repeated in the presence of POES (because resistance was seen or if results were not obtained in 3 weeks) for less than 1% of the isolates.

Because of problems encountered with PZA testing, two other methods have been developed. PZA is a synthetic analogue of nicotinamide, and strains of *M. tuberculosis* that are resistant to PZA are also resistant to a high concentration of nicotinamide (78). Based on this information, Brander developed a PZA susceptibility test using Lowenstein-Jensen medium at neutral pH containing 5 mg/ml of nicotinamide (79). This method has been found to be a useful and reliable assay for determining PZA resistance in some European countries (76), particularly when acid pH methods have been found to be problematic. Another alternative approach to detect resistance to PZA uses the determination of pyrazinamidase (PZase) activity (an enzyme that activates the PZA pro-drug into the metabolically active pyrazinoic acid) (76, 78, 80). Miller and colleagues (81) attempted to validate this approach by comparing the results of the radiometric susceptibility test with PZase activity for 428 strains of *M. tuberculosis*. In this study, all PZA-susceptible strains were PZase-positive; conversely, over 99 % of the PZA-resistant strains were PZase-negative. However, 0.8 % of the strains that grew in the presence of PZA were PZase-positive, and thus were thought to be falsely resistant in the radiometric assay. Further studies have shown that the frequency of mutations in *pncA*, the gene encoding PZase, ranged from 72-98% in isolates that were resistant to PZA in the radiometric assay (26, 32, 33, 34, 82). This wide range suggests that, for some strains, the radiometric assay may have resulted in demonstration of false-resistance, perhaps, as mentioned above, due to a large inoculum size, the presence of BSA, or contamination with another species.

3.4. Proportion method for first-line drugs using solid media

The proportion method uses the seeding of drug-free and drug-containing solid media with equal quantities of two dilutions of a standardized inoculum. Separate, countable colony-forming units (CFU) should be present for at least one of the dilutions on drug-free media. The number of CFU found on drug-containing media compared with those on drug-free media is expressed as a percentage. Strains of tubercle bacilli showing growth on drug-containing media that exceeds a certain proportion (greater than 1 %) of the growth encountered on drug-free media are considered resistant to that agent (39, 40, 83, 84). The critical concentrations of drugs have been established utilizing the MICs of wild type strains on this medium (27, 28, 39, 40, Table 1). The advantage of this technique is that the inoculum size can be estimated from the CFU counts. The disadvantage is that, if bacterial suspensions are not well dispersed, a single CFU could have arisen from a clump of bacilli rather than from an individual cell, resulting in an inaccurate calculation of the proportion of resistant mutants in the population.

The proportion method for mycobacterial susceptibility testing, developed in the 1960s (38, 39, 40, 85, 86, 87, 88, 89), has undergone various modifications (90), with a standard method published by the NCCLS in 2003 (27). Others (28, 91, 92) have also described the modified proportion method. The preferred medium in the U.S. for the proportion method is Middlebrook 7H10 agar plates, because its composition is fairly simple and easy to prepare, and the transparency of the medium allows for early detection and quantitation of colonies (27, 90). However, 7H10 agar must be incubated in a CO_2 environment, and the oleate-albumin-dextrose-catalase (OADC) supplement is expensive. Alternatively, use of Loewenstein-Jensen medium is recommended by the WHO and the IUATLD for surveillance studies on drug resistance in tuberculosis (93, 94). Drugs used in the proportion method can be prepared from reference powders or, when 7H10 agar is used, they can also be added in the form of drug impregnated disks (Table 1). The proportion method can be used to confirm resistance seen in the radiometric assay, as well as to test, for example, the following second-line (and newer) drugs: amikacin, capreomycin, cycloserine, ethionamide, kanamycin, p-aminosalicylic acid, ofloxacin, and rifabutin. However, because this method is not rapid, the standard of practice for first-line drug susceptibility testing in the U.S. and other high-income countries should be a broth-based method with a shorter incubation time (27).

3.5. Classical second-line and newer drugs

With the recent global resurgence of tuberculosis and especially MDR TB (93), there is an urgent need for the rapid determination of the susceptibility of *M. tuberculosis* isolates to drugs other than the primary agents. Data on critical concentrations for classical second-line and other newer drugs, tested either radiometrically or on 7H10 agar or Loewenstein-Jensen medium, are largely summarized in Table 1 (28, 54, 95, 96, 97, 98, 99). In a large multi-center study involving six major sites in the U.S., Canada, and Europe, the susceptibilities of 272 strains of *M. tuberculosis* to capreomycin, cycloserine, ethionamide, kanamycin, amikacin, ofloxacin, and rifabutin were determined radiometrically and compared to the results obtained with 7H10 agar method (100). The results from the study suggested that, with the exception of cycloserine, the radiometric method is capable of generating reliable susceptibility results for several second-line and newer drugs. Based on those results, the critical concentrations for the second-line and newer drugs in the radiometric system have been established (28, 100, Table 1).

3.6. Quality control for susceptibility testing methods.

Susceptibility testing is only of value when it is quality controlled for accuracy. In a 25-year study designed to assess reproducibility and drug stability, cooperating public health and clinical laboratorians in California performed drug susceptibility tests on *M. tuberculosis* using a uniform method, with standardized concentrations of antimycobacterial agents and a single type of medium (101). Beginning in 1963, stock drug solutions were distributed to the participating laboratories every year, and the stability of the stock drug solutions was examined yearly for 25 years. No deterioration was detected in INH or EMB solutions stored at 4^{o}C (for up to 1 year) at any time during the 25 years of the study, and there was only one instance of a slight change in the SM stock solutions. However, stock solutions of RMP did exhibit some changes over time: a 1% solution of RMP kept at 4^{o}C showed minor deterioration at 4 months, while a 5 % solution contained some precipitate at 1 month. In addition, when DMF-solubilized RMP was diluted in warm distilled water and stored, a precipitate formed. Therefore, each time that RMP medium was made, a fresh solution was diluted in warm distilled water, and added immediately to the medium.

In the U.S., standards for quality assurance in laboratory medicine have been greatly influenced by federal legislation (Clinical Laboratory Improvement Act of 1988--CLIA). The CLIA standards require that reference strains with known susceptibility patterns be tested with each new batch of drug and / or media. In addition, quality control tests should be performed at least once a week in laboratories that perform tests daily or weekly, or whenever a patient isolate is tested, if tests are performed less frequently. The H37Rv (ATCC 27294) or H37Ra (ATCC 25177) strains of *M. tuberculosis*, or another well-characterized strain that is susceptible to all standard antituberculosis agents, is commonly used for quality control purposes. For routine quality control testing, each laboratory should establish the concentrations of the drugs to be used, based on the level of susceptibility of each control strain. The highest concentration used should be the critical concentration of the drug, followed by at least three to four lower dilutions, such that the control strain grows in the two lowest dilutions (28). Variations from such a pattern will quickly alert the laboratory to problems with the control strain or the drug concentrations. New batches of drugs or media should be tested with a susceptible control strain before use with patient samples. The recommendations of the manufacturer should be followed for reconstitution of the lyophilized drugs, with aliquots kept at –80^{o}C for up to 6 months. In addition, media should always be checked for sterility and for the ability to support adequate growth before use.

Mutants of H37Rv selected for in vitro resistances to single and/or multiple drugs are also available; however, these strains are resistant to very high drug concentrations, and are not particularly suitable for quality control purposes. The use of resistant patient isolates that may be highly virulent is discouraged for reasons of biosafety. The approach outlined above, use of a single susceptible strain tested at various drug concentrations, will allow detection of any subtle deviations in the assay. Suspensions of quality-control strains may be prepared in 1-ml aliquots (single use) in BACTEC diluting fluid or Middlebrook 7H9 broth with 15% glycerol and frozen at -80^{o}C for up to 6 months.

Guthertz and colleagues (102) demonstrated the importance of controlling for all components of solid media, including lots of Middlebrook 7H10 agar, glycerol,

and, especially OADC. Of the components tested, seven of 23 (30%) lots of OADC, two of 13 (15%) lots of 7H10 agar, and zero of five lots of glycerol were found to be unacceptable. Griffith and Bodily (101) studied the stability of antimycobacterial drugs incorporated into 7H10 medium following storage at 4°C and 37°C for 1 month prior to inoculation with a control strain. The three drugs tested, SM, INH, and EMB, remained relatively stable in the cold; however, as expected, storage at 37°C resulted in a loss of activity that varied for each drug. A logical approach would be to use freshly prepared media and to not incubate at 37°C for longer than 3 to 4 weeks.

3.7. Accuracy in drug resistance testing

It is important to emphasize that efforts to shorten turnaround times should not be allowed to compromise the accuracy of the procedures. Based on laboratory findings, the patient's therapy may be altered to include more toxic and less effective compounds, and the duration of treatment may be extended. Therefore, laboratory results must be accurate. In 1994, the CDC reanalyzed eight nosocomial outbreaks of MDR TB and found evidence that 26% of the patients did not in fact have MDR TB (103). In a more recent study by the Los Angeles County Department of Health Services (104), it was concluded that susceptibility results should not be the only criteria used to dictate treatment. In that study, 13 % of the patients were considered to have been misdiagnosed as having MDR TB. The following problems were found: cross-contamination of the specimen with *M. avium* complex; suspected specimen mislabeling; successful treatment using drugs to which the isolate was reportedly resistant; discrepant susceptibility results on additional sputum specimens submitted by the patient; and, most significantly, no clinical evidence of tuberculosis. Therefore, careful clinical correlation is necessary in determining the efficacy of treatment and/or in making the diagnosis of MDR TB.

To ensure accuracy of in vitro susceptibility testing results, it is good practice to confirm all drug resistance in *M. tuberculosis* either by a second method or by a second laboratory. However, this confirmatory step should not delay reporting detection of resistance to the health care provider and to public health officials. When resistance is seen in the radiometric assay, it is good laboratory practice to repeat the assay for the particular drug, and also to perform the agar proportion method (considered the "gold standard") for all first-line drugs (confirming the radiometric results) and for additional (second-line) drugs.

If test results indicate that an isolate is resistant to PZA, especially if it is resistant to PZA alone, that isolate's identity should be confirmed, since *M. bovis* or *M. bovis* BCG are PZA-resistant, whereas the vast majority of *M. tuberculosis* isolates that are susceptible to other drugs are also susceptible to PZA (18, 105). However, PZA-monoresistant *M. tuberculosis* does occur (82, 106), and should be differentiated from *M. bovis* or *M. bovis* BCG. This is especially important if the laboratory routinely identifies isolates only to the level of the TBC. To differentiate the members of this complex, other assays, such as HPLC, colony morphology on LJ slants, niacin accumulation, nitrate reductase, TCH susceptibility, oxygen preference, and the recently developed deletion analysis, should be used (18, 90, 107, 108, 109). Due to the needs for accurate identification of the isolate, stringent quality control measures, and performance of a minimum number of tests to maintain proficiency, drug susceptibility testing should be performed primarily at larger hospitals or public health reference laboratories. This centralization of service also results in more accurate reporting of drug resistance, and immediate awareness of resistance trends.

4. NEW OR REDISCOVERED METHODS: INHIBITION OF GROWTH AND/OR METABOLISM IN THE PRESENCE OF DRUG

Because of the slow growth of *M. tuberculosis* (20 to 24 h generation time) and the need for immediate susceptibility results, a number of rapid methods have been developed in recent years. At present, many of these assays are still in the developmental stage. Some assays are based on drug-related inhibition of growth and/or metabolism, and these assays require a comparison between suspensions of tubercle bacilli in the presence and in the absence of drug. Thus, the inoculum and critical concentrations of drugs are still important parameters that need to be optimized for each assay.

4.1. Inhibition of growth

The Mycobacteria Growth Indicator Tube (MGIT), as developed by BD Diagnostic Systems (Sparks, MD), contains a modified 7H9 broth in conjunction with a fluorescence quenching-based oxygen sensor (silicon rubber impregnated with ruthenium pentahydrate). When this method was used in a multi-center study to evaluate susceptibility to the four SIRE drugs, in a comparison to the radiometric assay, it was found that there was no statistically significant difference between the susceptibility testing results of the two methods (110). However, as discussed above for the radiometric method (Section 3.2), results obtained with the MGIT system have suggested that more studies are needed to solve problems associated with susceptibility testing for SM and EMB (110, 111). The distinct advantage of the MGIT system is the elimination of use of the radioisotope ^{14}C. This assay is commercially available in both manual and automated formats, and MGIT susceptibility assays have recently received FDA approval.

The ESP Culture System II (Trek Diagnostic Systems, Inc. [formerly Difco], Westlake, OH), a fully automated continuously monitoring system for growth of mycobacteria, has been available for commercial use for a few years. The technology of the ESP II is based on detection of pressure changes (i.e., either gas production or gas consumption due to microbial growth) within the headspace above the broth culture medium in a sealed bottle (112). This device has received FDA approval for testing susceptibility to INH, RMP, and EMB, and has recently been reported to be capable of generating rapid and reliable PZA test results (113).

4.2. Detection of metabolic changes

Viability assays based on the use of indicator dyes have been used to determine the drug susceptibility status of mycobacteria. In 1958, Pital and colleagues used resazurin as an oxidation-reduction indicator, enabling them to report susceptibility test results for SM and INH within 5 to 8 days (114). Almost 40 years later, Yajko and coworkers (115) revisited use of this concept and compared the Alamar blue dye assay to the agar proportion method, finding an overall agreement in 194 out of 200 tests (97% agreement). In a more recent study, resazurin was used to detect MDR TB (116): 7 day MIC results for INH and RMP agreed with those results obtained from the agar proportion method for 80 clinical isolates. Another dye, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), is reduced by dehydrogenases in living cells to produce insoluble purple MTT formazan crystals, which after solubilization can be measured spectrophotometrically. This assay has been adapted for use in determining viability of *M. tuberculosis* cells following interaction with RMP (117, 118). It has been suggested that these simple, fairly rapid, and inexpensive screening methods could be used in countries where tuberculosis is a major health problem, and where funds for more expensive assays are not available.

Semi-quantitative analyses measuring products of metabolism have also been developed. Flow cytometry, comparing the DNA content and number of mycobacterial cells after 1 - 3 days in the presence or absence of drug, has been used to determine susceptibility of tubercle bacilli to antimycobacterial agents (119, 120). In addition, relatively rapid assessments of mycobacterial viability in the presence of drugs have been performed using bioluminescence that measures mycobacterial ATP levels (121, 122).

4.3. Bacteriophage methods

The luciferase reporter mycobacteriophage technique has been shown to be capable of distinguishing drug-resistant from drug-susceptible strains of *M. tuberculosis* in a 48-h assay (123). The percentage of residual light activity reflects the percentage of the mycobacterial population that is viable and able to support replication of the infecting bacteriophage, and consequently expression of the phage-encoded luciferase gene. Bacterial cells that are capable of producing light in the presence of a specific drug are determined to be resistant to that drug (123, 124, 125). One modification of this assay utilizes a Polaroid film box called the Bronx Box as a rapid and simple means for photographic detection of light from lytic phage-infected *M. tuberculosis* (124).

The PhaB (phage amplified biologically) assay is based on the ability of viable M. tuberculosis bacilli to protect infecting mycobacteriophage from chemical inactivation, and thus to support the replication of the infecting phage (126). Progeny mycobacteriophage released following lysis of mycobacterial cells are detected by infection and subsequent lysis of the rapidly growing sensor strain, *M. smegmatis*. Use of the PhaB assay to detect growth in the presence of a drug demonstrated that the assay identified the correct drug susceptibility in the majority of isolates, with results obtained within 3 to 4 days. In a recent comparative study on 133 clinical isolates of *M. tuberculosis* from South Africa, the commercial product FASTPlaqueTB-RIF^{TM} (Biotec Laboratories LTD., Ipswich,UK) was reported to provide RMP susceptibility results comparable to the BACTEC 460 within 2 days without the need for specialized equipment (127).

4.4. Evaluation of RNA levels

Detection of ribosomal RNA by commercially available DNA probes can be used as an early index of the metabolic activity of *M. tuberculosis*, because the photometric light units (proportional to the amount of hybridized probe) can be correlated with the numbers of CFU / ml (128). High photometric light unit readings after incubation in the presence of the drug (at a level similar to that seen without the drug) would indicate resistance.

Bacterial messenger RNA (mRNA) is typically short-lived, with a half-life of only a few minutes. Thus, it was reasoned that a semi-quantitative mRNA assay would be likely to detect only living organisms, and could be used as a direct indicator of response to therapy, as well as an indirect indicator of susceptibility to drugs. A novel reverse-transcriptase strand-displacement amplification system was developed for the detection of *M. tuberculosis* alpha-antigen mRNA directly in patient sputum samples (129). The loss of detectable mRNA corresponded to a rapid drop in the number of viable organisms present in each sputum sample over the first 4 days of treatment. Potentially, this assay would allow the patient to be rapidly and effectively monitored early in the treatment regimen. However, a caveat to this approach was demonstrated by a recent study. It is well known that *M. tuberculosis* can persist in humans in an altered physiological state for many years after initial infection, with the possibility of reactivation to active disease. In 2000, Hu and colleagues demonstrated that, although they are non-culturable, both microaerophilic stationary-phase *M. tuberculosis* treated with a high dose of RMP in vitro and PZA-induced persistent bacteria in mice still have transcriptional activity (130).

5. THE GENETIC APPROACH TO DRUG RESISTANCE DETECTION

5.1. Mycobacterial defenses against antibiotics

The mycobacteria are naturally resistant to several agents that are highly effective in killing other bacteria. The mycobacterial cell wall is composed of tightly packed long-chain (mycolic) fatty acids that provide the cell with a thick hydrophobic exterior coat (reviewed in 131). This unique cell wall exhibits significantly reduced permeability to several antibacterial agents, especially those that are large and hydrophilic. In addition to the relatively impermeable cell wall, degrading and drug-modifying enzymes, such as beta-lactamases, are produced and have been shown to be present in the periplasmic space (reviewed in 33).

As previously mentioned, resistance of *M. tuberculosis* to agents with proven antimycobacterial

capabilities is acquired during drug therapy. This acquired resistance develops when random chromosomal mutational events occur in a gene encoding a drug target or a drug-activating enzyme. If treatment includes a drug whose target or activating enzyme has been altered, these mutant cells will survive, and will eventually become the predominant population, unless other drugs active against the strain are also present. There is no evidence that genes or plasmids transferred from other organisms play a role in resistance to antituberculosis drugs. However, a mutation that allows the tubercle bacilli to survive in the presence of a drug may confer a disadvantage to the bacterial cell. For example, many drug-resistant strains of *M. tuberculosis* have extended generation times, and some lack critical enzymatic activities (36, 132, 133, 134, 135). Also, DNA cluster studies from the Netherlands have suggested that INH-resistant strains may be less likely to generate secondary cases (136). However, contradictory data from laboratory-based, and population-based, studies suggest that the impact of drug resistance-associated mutations on the fitness, survival, and transmissibility of *M. tuberculosis* may be achieved through a heterogeneous range of mutations, each with a different effect on phenotype (reviewed in 137).

5.2. Drug targets differ for each antimycobacterial agent

Over the past 10 years, significant progress has been made in determining the biochemical targets for each of the antimycobacterial drugs (reviewed in 26, 32, 33, 34, 138, 139). The good news is that no single mutation in *M. tuberculosis* results in resistance to more than one first-line drug, and no single genetic alteration has yet been found that results in the MDR phenotype. Instead, MDR TB results from step-wise accumulation of individual mutations. The bad news is that the differing locations for the mutations have made detection of multi-drug resistance by molecular methods more challenging. Furthermore, resistance to certain drugs can involve a mutation in one of several possible genes or gene complexes. These problems can be significant when attempts are made to develop and validate rapid and accurate assays for resistance to multiple drugs, and they indicate that present-day molecular detection of drug resistance is far from ideal.

For example, resistance to INH is difficult to detect using molecular methods since mutations can occur in more than one locus. Resistance-associated mutations have been found in the mycobacterial catalase gene (*katG*) (catalase activates the INH pro-drug), or in genes encoding enzymes that participate in synthesis of the cell wall mycolic acids (ie. *inhA*, *kasA*) (32, 33, 34, 139, 140, 141, 142, 143, 144). However, approximately 15 to 25% of INH-resistant isolates do not contain mutations in any of these regions, suggesting that other targets must also be involved in resistance to this drug. Likewise, only about 70% of EMB-resistant clinical isolates of *M. tuberculosis* contain mutations in *embB*, the drug target involved in biosynthesis of the cell wall component, arabinan (33, 63, 64, 139, 145, 146). Other genes involved in EMB resistance are unknown. RMP resistance is more easily assessed using molecular methods since >96% of the RMP-resistant isolates of *M. tuberculosis* contain mutations in a well-defined, 81-bp (27-codon) central region of the gene encoding the beta-subunit of RNA polymerase (*rpoB*) (24, 32, 33, 147, 148). Similarly for PZA-resistance, up to 97% of all PZA-resistant clinical isolates tested carry a mutation in the putative promoter region or the structural gene (*pncA*) for PZase, the enzyme responsible for activation of the PZA pro-drug (18, 33, 149, 150, 151). For a more detailed and comprehensive review and discussion of the molecular mechanisms involved in drug resistance, see Wade, M. M. and Y. Zhang 2004. Drug resistance mechanisms in tuberculosis. Frnt. Biosci. 9: 975-994.)

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5.3. Pros and cons of molecular methods

There are several advantages to the use of molecular amplification methods for the detection of drug resistance in *M. tuberculosis*. Sample preparation begins with release of DNA from mycobacterial cells and amplification of the relevant region of the target gene. Amplification procedures can be performed directly on clinical specimens if a sufficient quantity of bacterial cells is present. Thus, biohazard risk in the laboratory is reduced, turnaround time is rapid, and therapy can be optimized before the culture results are available. Secondly, following identification of the mutation, the genotype of the isolate may be more informative than are the phenotypic results obtained by conventional susceptibility testing. In addition, because mono-resistance to RMP is rare, the molecular determination of RMP resistance is a rapid and sensitive means for detection of MDR TB.

In general, disadvantages of molecular methods include the following: low sensitivity if too few or mixed populations of organisms are present (especially when the methods are used directly on clinical specimens), false-positive results due to contamination with previous amplification products, and lack of standardized assays or FDA-approved commercial products. Also important is the fact that the presence of a heterogeneous bacterial population, composed of both resistant and susceptible cells, will make the clinical interpretation of molecular methods difficult (36, 152, 153, 154). A problem specific to the testing of *M. tuberculosis* is the large number of assays required for detection of drug resistance to first-line drugs alone. At present, there are possibly three or more known targets for INH, one for RMP, one for PZA, possibly more than one for EMB, and two for SM. Each assay may require sequencing a large region of DNA after selection of appropriate primers and optimization of the amplification reaction, followed by determination of the appropriate mutation detection method, and finally, validation studies with well-characterized strains and a sufficient number of patient specimens (32, 33).

5.4. Multiple methods for detection of mutations

Selection of the method used for detection of mutations associated with drug resistance in *M. tuberculosis* can be challenging (reviewed in 32, 33, 34, 155, 156, 157). Since DNA sequencing of the amplified product not only detects but also identifies the specific mutation, this method serves as the "gold standard." DNA sequencing also differentiates between mutations resulting

in an amino acid change and mutations that are silent. Most of the non-DNA sequence-based mutation detection methods are unable to make this distinction. However, use of DNA sequencing for the precise detection of mutations is labor intensive, and requires not only expensive equipment but also a high level of expertise.

In most mutation-detection methods that do not use DNA sequencing, validation of assays has usually begun by testing for RMP resistance, because of the clinical significance of resistance to this drug and its association with MDR TB, and because close to 100 % of resistance is due to mutations in the 81-bp region of a single target gene. The assays are based on DNA and RNA research techniques, and some amplification method is used, most often the polymerase chain reaction (PCR). The PCR product can be tested for resistance-associated mutations by digestion with restriction endonucleases followed by gel analysis to detect fragment size differences (restriction fragment length polymorphisms, or RFLP), or by direct gel analysis to detect single strand conformation polymorphisms (SSCP) caused by DNA sequence changes that result in structural changes. Both of these approaches require use of the wild type susceptible strain as a comparison control. Another successful approach is to detect mutations using hybridization, either with allele-specific nucleic acid probes or with peptide nucleic acid (PNA) probes in ELISA-based methods (158). The most rapid and powerful new method, real-time PCR, uses hybridization with fluorescence-labeled probes or molecular beacons during amplification, which can be followed by fluorescence-probe melting profiles to detect resistance-associated mutations (34, 155, 156, 157, 159, 160, 161).

Another promising development is the use of oligonucleotide, or DNA, micro-arrays. Micro-arrays consist of a small solid surface made of glass, silicon, or other material that serves as a platform for anchoring a large collection of fragments of DNA (oligonucleotides on DNA chips, cDNA fragments on DNA arrays) (34, 156, 157, 162, 163, 164). These DNA probes are used to capture (by hybridization) complementary sequences of DNA or RNA in the sample being analyzed. For hybridization to DNA chips, the DNA or RNA target in the sample must be amplified, and labeled with a fluorescent dye. Following hybridization of the labeled sample onto the micro-array, the hybridized strands are detected by excitation of the fluorescent tags with a laser, and the emission is detected with a scanning fluorometer. Complete sequence matches yield bright patches of fluorescence, and single-base mismatches produce a dimmer signal. Because of the capability of the micro-arrays to contain hundreds of thousands of probes, a single chip can be used to identify the species of *Mycobacterium*, and to detect mutations in all known target genes for antimycobacterial drugs. The results from preliminary testing of one system (GeneChip; Affymetrix, Santa Clara, CA) for mycobacterial identification and detection of resistance to RMP, using regions of the 16S rRNA and rpoB genes, respectively, have been promising (165, 166).

An alternative approach is to assess whether mutations in a gene have interfered with enzymatic activity of a protein required for drug activation. Suzuki and colleagues (167) used PCR to amplify *pncA*, and then synthesized PZase using an in vitro transcription-translation coupled system. The measured PZase activity was then compared to that of the wild type gene. This system has the advantage of eliminating the need for growing a large biomass to test for PZase activity, and it also eliminates the need for DNA sequencing.

6. VALIDATION OF NEW METHODS FOR DETECTING DRUG RESISTANCE

The newer methods described above have been developed as part of an ongoing quest for improved assays that are more rapid and accurate. Following the development of PCR by Mullis and colleagues in 1986 (168), various companies developed nucleic acid amplification protocols (169) for diagnostic purposes, which in turn were tested in numerous clinical trials. Initially, the results of the novel kits were compared to the conventional gold standard, the culture. However, there were many discrepant results, and ultimately the gold standard was redefined to be a clinical diagnosis that includes patient outcome "confirmed" by one or more laboratory tests (170, 171, 172, 173). This novel approach requires that both laboratorians and clinicians be involved in the evaluation of new diagnostic methods, especially those developed for the detection of drug resistance in the slow-growing *M. tuberculosis*.

Ideally, for the validation of a new method, a large number of strains (40, 174), including both drug-susceptible and -resistant, should be tested at several independent laboratories, and the results compared with a previously validated method. In addition, the genotypes of those strains should be known, i.e., they should be identified as susceptible or resistant, and if resistant, the mutation(s) should be identified. Furthermore, to prevent any bias in the study, it is important that the strains be shown to be genetically dissimilar, through DNA fingerprinting. Only a few institutions have access to such well-characterized strains; therefore, an ideal situation would be the establishment of a repository of frozen strains with aliquots available to evaluating laboratories. This approach would allow different procedures to be compared over time, in different laboratories, using the same group of well-characterized strains.

7. PERSPECTIVE

It has been predicted that about 79 million deaths from tuberculosis will occur in the next three decades (175). However, mathematical model formulation in the biomedical sciences often emphasizes what is unknown, rather than providing a predictive tool for future events (176, 177). This is clearly the case for tuberculosis, where uncertainty still surrounds essential aspects of transmission and pathogenesis. New molecular and genetic research tools (15, 160, 168, 171, 178, 179, 180, 181) offer great promise in the resolution of many of these unknowns. In the poorest regions of the world, where tuberculosis is most prevalent, the HIV epidemic is complicating successful

outcomes. Thus, in such areas of high or increasing HIV prevalence, spread of the virus must be addressed before gains can be made in limiting the transmissibility of *M. tuberculosis* (176, 182, 183, 184).

Furthermore, in confronting drug resistance in tuberculosis, it is not surprising that the priority for susceptibility testing differs between a high TB-prevalence, low-income country, and a low TB-prevalence, high-income country. In the 1960's, both the IUATLD (185) and the WHO (186) questioned the value of performing susceptibility testing before the initiation of TB chemotherapy in high-prevalence, low-income countries. Fortunately, low TB-prevalence, high-income countries do recognize the importance, and usually can afford, to test each newly isolated strain of tubercle bacilli, whether from a new case or a recurrence (187, 188). In addition to the direct benefit to the individual patient, routine testing rapidly recognizes the unexpected appearance and spread of increased numbers of drug-resistant cases. However, in high TB-prevalence, low-income countries, routine susceptibility testing cannot be implemented, often due to lack of funds and technical expertise. To determine the incidence of resistance in these situations, surveys of primary and acquired drug resistance such as the WHO and IUATLD Global Project on anti-TB drug resistance surveillance should be performed (23, 189). Information from such surveys has been useful in defining chemotherapy regimens for national programs. However, the results of resistance screenings are useful only if surveys are performed using standardized bacteriological methodology, and tests are performed in national or regional laboratories monitored by an international system of proficiency testing (23, 190). A well-defined sample population is also important since increases in drug-resistance rates in low-prevalence, high-income countries can be due to inclusion of high numbers of tuberculosis patients immigrating from high-prevalence, low-income countries (reviewed in 191).

Trends analysis from those countries that have had more than one survey done over the years clearly demonstrated the importance of sound TB control practices in preventing the emergence of drug resistance (192). Especially when high levels of drug resistance are found, strict management of MDR TB cases within a proper direct observed therapy (DOTS) setting is needed to contain transmission and reduce the high burden imposed by this disease. In addition to effective TB control, the clinical laboratory plays a vital role in the global commitment to eliminate tuberculosis (190). The ultimate goal for the laboratory is the provision of timely, appropriate, and adequate service that is continually evaluated and updated. A highly infectious tuberculosis patient should have access to state-of-the-art laboratory services that support appropriate TB control efforts, even if the patient resides in an area where a local laboratory is not capable of providing those services. Total dependence on results that are provided 2 - 4 weeks (or even months) later by the conventional "gold standard" susceptibility methods may not be acceptable for successful patient outcome. The slow growth of the pathogenic mycobacteria should no longer dictate the turn-around-time for susceptibility results. In many environments, rapid molecular testing can play a significant role in the clinical management of drug-resistant tuberculosis. Several approaches have been used to successfully detect and identify the most common mutations associated with drug resistance. In many instances, knowledge gained from determination of the particular mutation can provide significant information on drug resistance, the level of resistance, cross-resistance to a similar drug, relatedness of strains, and infectivity. Innovative ideas, such as centralization of testing at large public health laboratories, may be necessary to move toward more rapid testing, especially using molecular methods. However, the benefits of providing new and more clinically relevant assays to a larger population, especially in high-incidence regions of the world, would be of great public health significance to all populations.

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