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Drug Susceptibility Testing of *Mycobacterium tuberculosis* Complex by Use of a High-Throughput, Reproducible, Absolute Concentration Method[▽]

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Accurate drug susceptibility testing (DST) for *Mycobacterium tuberculosis* is highly important for both therapy guidance and surveillance of drug resistance. Although liquid medium DST methods are used increasingly and seem most efficient and fast, the high costs hamper widespread implementation. In addition, an inability to check the colony morphology of the growing bacteria is a disadvantage of these methods. Moreover, these methods discriminate only between susceptibility and resistance and do not determine the MIC. In this paper, we describe a low-cost, reproducible, high-throughput, proportional absolute concentration DST method. The method uses a concentration series of antituberculosis drugs, including pyrazinamide in 7H10 medium, distributed semiautomatically in 25-well plates. The performance of this 25-well DST method was evaluated by the World Health Organization and the International Union against Tuberculosis and Lung Disease in 10 rounds of proficiency testing regarding sensitivity, specificity, efficiency, reproducibility, and predictive value for resistance and susceptibility. The performance of the method for these characteristics was 100% for isoniazid and from 96 to 100% for rifampin, 91 to 100% for streptomycin, and 85 to 100% for ethambutol. The method was 100% reproducible for all four drugs. The levels of drug resistance and the MIC distributions for the first-line antituberculosis drugs were determined for all 7,956 *M. tuberculosis* strains isolated in The Netherlands from 1998 to 2005 and amounted to 7.5% for isoniazid, 1.4% for rifampin, 8.5% for streptomycin, and 1.0% for ethambutol. Pyrazinamide testing was successful for 7,026 (88.3%) of the isolates and showed a resistance level of 0.8%.

For both guidance of therapy and surveillance of drug resistance, accurate drug susceptibility testing (DST) for *Mycobacterium tuberculosis* complex is considered highly important (1). In the 1950s, Cannetti et al. described the first DST method for *M. tuberculosis*, involving the preparation of a concentration series of drugs against *M. tuberculosis* complex in Löwenstein-Jensen medium, inoculation of the bacterial cultures on the slants, and reading of the inhibition of growth by drugs at different concentrations (3). Although many laboratories still use this method, several alternative DST methods have become available in due time, including those that use impregnated discs (28) or strips (the Etest) (7, 27), the radiometric BACTEC 460TB method (16, 17), the automated nonradiometric Mycobacteria Growth Indicator Tube (MGIT) 960 system (13, 19), the colorimetric test, and the microtiter-based Alamar Blue assay (6).

In contrast to those for Alamar Blue and the Etest, the BACTEC and the MGIT methods have been adopted by many laboratories in the Western world (2, 14, 15, 19, 29). These methods are based on automated detection of growth in a liquid medium with antituberculosis drugs and therefore are faster than methods based on visual detection of growth on solid medium. However, one disadvantage of these systems is the lack of possibility to check the colony morphology of the bacterial cultures. Both invisible contamination and overgrowth with atypical mycobacteria affect the reliability of the tests (15, 19). In addition, these methods have disadvantages in that they involve multiple tubes or bottles and are restricted to discriminating between resistance and susceptibility, rather than determining the exact MICs of the drugs. Although in the daily routine the automated liquid medium DST methods seem most efficient, the high costs hamper widespread implementation.

The World Health Organization (WHO) and the International Union against Tuberculosis and Lung Disease (IUATLD) facilitate the standardization and quality control of DST for antituberculosis drugs. A network of supranational reference laboratories has been established, and proficiency testing for isoniazid (INH), rifampin (RMP), streptomycin (SM), and ethambutol (EMB) is carried out on a regular basis (10, 11). This network and the proficiency tests are essential to the WHO/IUATLD global project on the surveillance of antituberculosis drug resistance (30). This project aims to determine global trends in the level of drug resistance and to explore trends in resistance over time.

Since 1993, all *M. tuberculosis* complex strains isolated in The Netherlands have been subjected to DST using the aforementioned drugs, within the framework of a national tuberculosis surveillance program (9). These drug susceptibility tests are performed by using a high-throughput, proportional absolute concentration method developed at the National Institute

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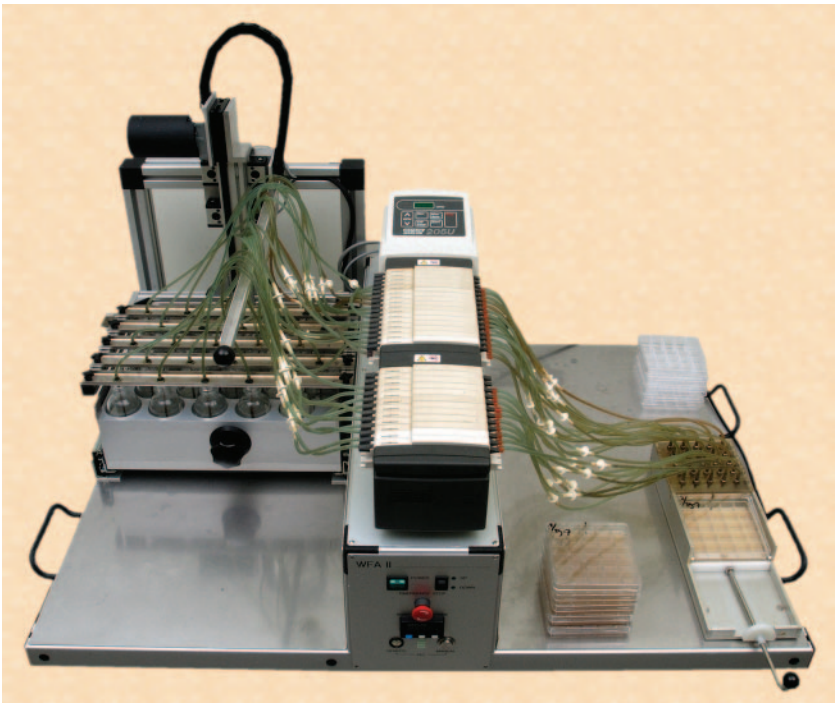

FIG. 1. Picture of the dispenser device for preparation of the 25-well DST plates. On the left, the bottles containing the liquid agar medium with the various concentrations of anti-TB drugs are shown. These bottles are kept in an iron heater to prevent the agar from solidifying. From each bottle, 2.5 ml medium is transferred through a plastic hose by a pumping device (middle) into one well of the 25-well plate (right). By activating the pump, the pump transfers 2.5 ml agar into all 25 wells at the same time. After a plate is filled, it is replaced by another empty plate and subsequently the pump is activated again.

for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. This method uses a concentration series of antituberculosis drugs in supplemented 7H10 medium distributed in 25-well plates, facilitating fast and easy preparation and reading of the plates. Furthermore, a control is included to check the sizes of the inocula and to compare the mycobacterial growth levels in the presence of different concentrations of antituberculosis drugs in a proportional way. This method enables a determination of the MIC, defined as the concentration that inhibits more than 99% of the inoculum.

In this paper, the 25-well absolute concentration DST method, including testing with pyrazinamide (PZA), is described in detail and its performance in the WHO/IUATLD proficiency-testing program is presented. In addition, the levels of drug resistance and the MIC distributions for the first-line antituberculosis drugs for *M. tuberculosis* in The Netherlands are presented for the period of 1998 to 2005.

MATERIALS AND METHODS

Principle of the test. Briefly, the test consists of the preparation of 25-well plates with solid 7H10 medium containing different concentrations of antituberculosis drugs in each well by using a homemade dispenser. The plates are subsequently inoculated by adding 10 μl *Mycobacterium* suspension to each well. After appropriate incubation, the MICs are assessed. In this paper, the method is described for testing the first-line drugs. However, when resistance against the first-line antituberculosis drugs is recorded, second-line drugs are tested in a similar way. The whole 25-well DST method is described in detail in a standard operating procedure that can be found on the Internet at www.caontb.rivm.nl.

Preparation of the 25-well DST plates. For all antituberculosis drugs except PZA, Middlebrook 7H10 medium (Becton Dickinson and Company, Sparks, MD) supplemented with oleic acid-dextrose-catalase (OADC; Becton Dickinson and Company) of pH 6.6 is used (28). To prepare 500 ml medium solution, the amount of Middlebrook 7H10 powder recommended by the manufacturer is added to 450 ml distilled water. Subsequently, 2.5 ml glycerol (about 87%; BDH Laboratory Supplies, Poole, England) is added and the solution is placed in a water bath at 100°C until the agar is completely dissolved. This solution is sterilized for 10 min at 121°C. After cooling to 50°C in a water bath, 50 ml OADC, preheated to the same temperature, is added. One milliliter of agar is solidified to check the final pH of the medium, which should amount to 6.6 ± 0.2.

For the preparation of about 40 DST plates, 2.5 liters of Middlebrook 7H10 medium, supplemented with OADC, is prepared. In 23 bottles, dilutions of antituberculosis drugs in this medium are prepared with the following concentrations: 0.1, 0.2, 0.5, 1, and 2 μg/ml INH (isonicotinic acid hydrazide; Sigma Chemical Co., St. Louis, MO); 0.1, 0.2, 0.5, 1, 2, and 5 μg/ml RMP (Sigma); 1, 2, 5, 10, and 20 μg/ml SM (streptomycin sulfate; Sigma); 1, 2, 5, 10, and 20 μg/ml EMB (ethambutol dihydrocloride; Sigma); 5 μg/ml amikacin (AMK; ICN Biomedicals, Inc., OH); and 1 μg/ml *p*-aminosalicylic acid (PAS; Sigma). The medium with antituberculosis drugs from these 23 bottles and from two bottles containing medium without drugs (for the control wells) is transferred in 2.5-ml amounts into 25-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) by using a dispenser developed at the RIVM (Fig. 1). With this device, the medium is transferred from the bottles into the plates through 25 small plastic hoses by an electronic pump. Figure 2 (left panel) shows a picture demonstrating how the different concentrations of drugs in medium are distributed into the 25-well plates. To solidify the medium, the plates are left at room temperature for approximately 1 h. The plates are stored at 4°C until use.

Because PZA testing requires growth medium of another pH (see below), separate DST plates are prepared containing 10, 20, 50, and 100 μg/ml of this drug. Before adding OADC to the dissolved 7H10 medium, the pH is lowered by adding 0.95 ml of 3 M HCl to achieve a final pH of 5.7 ± 0.15.

Inoculation and incubation of the DST plates. The *Mycobacterium* isolates to be tested are suspended by adding a small loop of bacteria in 40 ml distilled water. To ensure biosafety, the bacteria are suspended in unbreakable plastic bottles (Balis, Boven-Leeuwen, The Netherlands). The suspensions are homogenized by shaking with glass beads for 20 min at 350 rpm in a closed biosafety bucket (Lab-Line model 4626-ICE; Beun-de Ronde, Abcoude, The Nether-

INH 0.2	INH 0.1	AMK 5	C 1/100	C
RMP 0.2	RMP 0.1	INH 2	INH 1	INH 0.5
PAS 1	RMP 5	RMP 2	RMP 1	RMP 0.5
EMB 20	EMB 10	EMB 5	EMB 2	EMB 1
SM 20	SM 10	SM 5	SM 2	SM 1

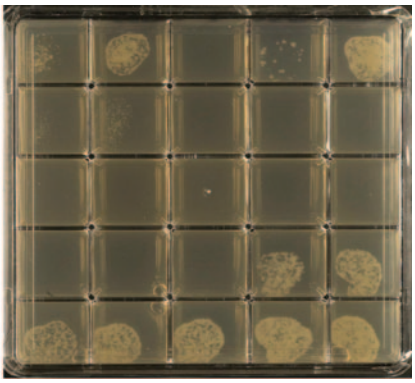

FIG. 2. Reading of a 25-well DST plate. The left panel shows the distribution of the four control wells and the 21 wells containing anti-TB drugs in the respective concentrations (μg/ml) in a DST plate. The wells representing the breakpoint concentrations are shown in gray. The control wells include undiluted bacterial suspension (C), the 1:100-diluted suspension (C 1/100), 5 μg/ml AMK, and 1 μg/ml PAS. The right panel shows a typical result of an incubated DST plate inoculated with an *M. tuberculosis* strain resistant to streptomycin and intermediate resistant to isoniazid. The MICs for this strain are 0.5 μg/ml for INH, 0.2 μg/ml for RMP, 5 μg/ml for EMB, and >20 μg/ml for SM.

lands). To allow sedimentation of coarse-grained particles, the suspensions are incubated at room temperature for 10 min. The turbidity of the supernatant is adjusted to a stock solution with a McFarland standard of 1 to obtain a density of 2×10^5 to 10×10^5 CFU/ml. If the suspension is too turbid, sterile water is added to dilute the suspension.

The DST plates are inoculated by adding 10 μl of the suspensions on 24 wells of the DST plates by using an automated (repetition) pipette (Socorex, Omnilabo, Breda, The Netherlands). The final well, one of the control wells (containing medium without any drug), is inoculated with a 1/100 dilution of the mycobacterial suspension, allowing the quantification/counting of the inocula after incubation. The plates are inoculated at 35.5°C in a CO_2 incubator with a water reservoir to prevent evaporation of the plates.

Reading of DST plates. The DST plates are checked for mycobacterial growth after 6, 12, and 19 days. The reading of the plates is carried out when the bacterial growth on the two control wells without antituberculosis (anti-TB) drugs is sufficient, i.e., when colonies are clearly visible. The MIC is the lowest concentration of antituberculosis drugs that inhibits more than 99% of the growth of the mycobacterial culture. The MIC can be determined by a comparison of the growth with the 1/100 control well. The interpretation of the MIC readings, i.e., the decision as to whether a strain is susceptible or resistant to a certain drug, is based on the breakpoint concentrations for the respective drugs. The breakpoint concentrations are 1 μg/ml for INH and RMP, 5 μg/ml for EMB and SM, and 50 μg/ml for PZA (Fig. 2). Growth at the breakpoint concentration is reported as susceptible, and growth at higher concentrations of drug is considered resistant.

For therapy guidance, isolates with MICs of 0.2 or 0.5 μg/ml for INH are reported as "intermediate resistant." For national and international surveillance of antituberculosis drug resistance, intermediate-resistant isolates are reported as "resistant."

PZA drug susceptibility testing. Since its synthesis in 1952 as an analogue of nicotinamide, PZA has appeared to be more active in vivo than the in vitro susceptibility seemed to indicate. Presumably, the main metabolite, pyrazinoic acid, which reaches even higher plasma levels than does PZA after an oral dose of PZA, contributes significantly to the activity of the drug in vivo.

Since the optimum activity of PZA in vitro is expressed under acid conditions, it has been propagated to perform susceptibility tests in/on acid media of pHs 5.0 to 5.5 (12, 20). At this pH range, the MICs on solid media for susceptible *M. tuberculosis* strains is usually 25 to 100 μg/ml; at a neutral pH, these values are at least 10 times higher (8). The problem is that at pH ranges of 5.0 to 5.5, mycobacteria grow very poorly or not at all (18). In this respect, the method of acidifying the medium was found to be an important factor (18, 20). Initially, PZA testing was usually carried out on Löwenstein-Jensen medium acidified with hydrochloric acid. In the search for more reliable results, Stottmeier et al. (18) developed a method using 7H10 agar of pH 5.5, with a specific ratio of buffering phosphate salts.

Study population. The study population consists of all 7,956 *M. tuberculosis* strains isolated in The Netherlands from January 1998 to December 2005. A single isolate was included for each culture-positive TB case per year. *Mycobacterium* isolates were sent to the National Mycobacteria Reference Laboratory at the RIVM by 43 peripheral laboratories in The Netherlands. About 55% of the respective tuberculosis patients were of non-Dutch nationality and originated from multiple high-prevalence areas. Nearly 80% of the *M. tuberculosis* strains with a form of resistance were isolated from these foreign-born patients.

Identification of mycobacterial isolates. *M. tuberculosis* complex strains were identified by using either the AccuProbe culture confirmation test (Gen-Probe, Inc., San Diego, CA) or, since January 2004, the GenoType MTBC assay (Hain Lifescience GmbH, Nehren, Germany). All *M. tuberculosis* complex isolates were subjected to IS*6110* restriction fragment length polymorphism (RFLP) typing for epidemiological investigations and for identification procedures (21). Before January 2004, *M. tuberculosis* complex isolates positive by Gen-Probe test and with a positive reaction by the nitratase test were identified as *M. tuberculosis*. All *M. tuberculosis* complex strains negative by nitratase test were subjected to RFLP typing with a probe complementary to the *mtp40* sequence (4). Nitratase-negative strains positive by *mtp40* hybridization were considered to be *M. tuberculosis* (about 8% of the *M. tuberculosis* isolates in The Netherlands). Nitratase-negative strains devoid of the *mtp40* sequence were identified as either *Mycobacterium bovis* or *Mycobacterium bovis* BCG, depending on their IS*6110* and IS*1081* RFLP patterns (23, 24). *Mycobacterium microti* and "*Mycobacterium canettii*" isolates were recognized by IS*6110* RFLP typing, and their identification was confirmed by spoligotyping (25, 26). In this study, *Mycobacterium africanum* was not distinguished from *M. tuberculosis*.

Quality control. Quality assurance of DST is ensured at four different levels. Of the wells of the 25-well DST plates, one contains 1 μg/ml PAS and one contains 5 μg/ml AMK, with both wells serving as a control to check the purity and identification of each of the isolates; in general, *M. tuberculosis* complex isolates do not grow on medium with these drugs. For each series of *M. tuberculosis* isolates inoculated on DST plates, one *Mycobacterium gordonae* strain, one *Mycobacterium avium* strain, and three *M. tuberculosis* control strains with known MICs are included as internal controls in each experiment. As a second-line quality control, strains are exchanged in a blind fashion with a peripheral laboratory and retested to test the reproducibility of the assay. As a third-line control, our laboratory participates in WHO/IUATLD DST proficiency testing (10).

RESULTS

For the vast majority of strains, an indicative result for drug susceptibility or resistance could be reported to the clinicians after 6 days. The final MIC results were determined for most isolates after 12 days.

Inoculum size. Since standardization of the inoculum in drug susceptibility testing of mycobacteria is important, in particular when using the generally recommended proportion method, the actual inoculum size was determined for a random sample of 244 cultures. These cultures varied in age, i.e., they

TABLE 1. Colony counts from 244 inocula of *M. tuberculosis* cultures with varying incubation times (age) before preparation of the inoculum

No. of CFU in 1/100-diluted inoculum	No. of cultures at indicated no. of days					Total no. of cultures
	<7	7–14	15–21	22–28	>28	
<20	13	12	3	2	7	37
21–40	33	30	16	3	4	86
41–60	32	32	10	7	1	82
61–80	11	5	3	1	1	21
>80	10	4	1	2	1	18
Total	99	83	33	15	14	244

had different times of incubation before preparation of the inoculum for DST. The inoculum size was determined by counting the number of colonies in the well inoculated with the 1/100-diluted inoculum at the time of reading of the MICs. As described in Materials and Methods, the inocula were aimed to be in the range of 2×10^3 to 10×10^3 CFU. Therefore, 1% of these should yield 20 to 100 colonies. It follows from Table 1 that this goal was indeed reached in the majority of the cases, with a tendency to yield less CFU from inocula of cultures that were more than 4 weeks old (Table 1).

Prevalence and levels of drug resistance. From 1998 to 2005, the average levels of resistance among 7,956 *M. tuberculosis* strains isolated in The Netherlands amounted to 7.5% for INH, 1.4% for RMP, 8.5% for SM, and 1.0% for EMB. In Table 2, the resistance against single antituberculosis drugs and combinations of drugs is depicted by year. In the 8-year study period, a slight increase was observed in the proportion of resistance to INH ($P = 0.02$). A slight decrease was observed in SM resistance ($P = 0.04$), combined resistance to INH plus SM plus EMB ($P = 0.03$), and any resistance ($P = 0.02$). The number of multidrug-resistant (MDR) isolates, defined as isolates with resistance to at least INH and RMP, was stable at around 1% (Table 2), except in the year 2003, when the number of MDR isolates was higher (17) because of an outbreak of MDR-TB (5). Thereafter, the level of MDR normalized to 10 in 2004 and to 7 in 2005.

The MICs recorded for all isolates are depicted in Table 3. The percentages of isolates growing at the breakpoint concentration varied significantly by drug and were 0.0% for RMP, 2.9% for SM, 4.7% for INH, and 45.0% for EMB. Most of the RMP-resistant isolates showed MICs of >5 μg/ml, and most of the EMB-resistant isolates had MICs of 10 μg/ml, whereas for INH and SM, a binominal distribution was recorded (Table 3).

Results of the 25-well DST method in the WHO/IUATLD proficiency testing. Our laboratory participates in the ongoing quality assurance program for drug susceptibility testing of *M. tuberculosis* in the WHO/IUATLD Supranational Laboratory Network (10). In this program, sensitivity (ability to detect true resistance), specificity (ability to detect true susceptibility), efficiency (ratio of the number of correct results and the total number of results), predictive value for resistance (rate of true resistance to total resistance), predictive value for susceptibility (rate of true susceptibility to total susceptibility), and reproducibility (intralaboratory agreement between duplicate

TABLE 2. Drug resistance in *M. tuberculosis* to single and combined anti-TB drugs in The Netherlands from 1998 to 2005

Drug[a]	*M. tuberculosis* drug resistance (%) in:							
	1998 ($n = 987$)	1999 ($n = 1,109$)	2000 ($n = 1,048$)	2001 ($n = 1,080$)	2002 ($n = 1,033$)	2003 ($n = 966$)	2004 ($n = 882$)	2005 ($n = 851$)
H	2.8	3.4	3.4	3.4	3.5	4.2	3.5	3.6
R	0.2	0.3	0.2	0.1	0.1	0.1	0.2	0.5
S	6.5	5.1	5.9	5.6	6.1	3.8	3.3	2.2
E	0.3			0.3	0.3			
H, R	0.2	0.2	0.5	0.3	0.2	0.4	0.3	0.1
H, R, S	0.3	0.3	0.1	0.5	0.1	0.5	0.5	
H, R, S, E	0.5	0.4	0.2	0.3	0.7	0.7	0.2	0.7
H, R, E		0.3	0.1	0.1		0.1	0.1	
H, S	2.4	3.7	3.7	2.9	1.6	2.5	2.3	2.1
H, S, E	0.6	0.2	0.3	0.2	0.3		0.1	
H, E	0.1		0.1		0.1	0.1		
R, S					0.1	0.2		0.1
R, E		0.1						
R, E, S						0.1		
S, E					0.1	0.2		
Total H	6.9	8.5	8.4	7.7	6.5	8.5	7.0	6.5
Total R	1.2	1.6	1.1	1.3	1.2	2.1	1.3	1.4
Total S	10.3	9.7	10.2	9.5	8.9	7.8	6.4	5.1
Total E	1.5	1.0	0.7	0.9	1.5	1.2	0.4	0.7
Any drug	13.9	14.0	14.5	13.7	13.2	12.9	10.5	9.3
No. of MDR isolates	10	12	9	12	10	17	10	7

[a] H, isoniazid; R, rifampin; S, streptomycin; E, ethambutol.

TABLE 3. MIC distribution for INH, RMP, SM, EMB, and PYR[a]

INH		RMP		SM		EMB		PYR[b]	
MIC (μg/ml)	% of isolates	MIC (μg/ml)	% of isolates	MIC (μg/ml)	% of isolates	MIC (μg/ml)	% of isolates	MIC (μg/ml)	% of isolates
≤0.1	87.8	≤0.1	15.5	≤1	72.3	≤1	2.2	10	40.2
0.2	**4.7**	0.2	72.5	2	16.2	2	51.9	20	47.0
0.5	2.0	0.5	10.6	**5**	**2.9**	**5**	**45.0**	**50**	**11.7**
1	0.6	**1**	**0.0**	10	4.3	10	0.8	100	0.3
2	0.4	2	0.1	20	1.4	20	0.2	>100	0.8
>2	4.5	5	0.1	>20	2.9				
		>5	1.2						

[a] Shown are distributions among 7,956 isolates of *M. tuberculosis* from 1998 to 2005 in The Netherlands. Breakpoint concentrations and percentages of isolates growing at those concentrations are in bold type.
[b] The number of isolates tested for PYR was 7,026; the value is lower because 930 isolates did not grow at a pH of 5.7.

cultures) are determined. "True" or judicial results ("gold standard") are considered to be the results obtained by the majority of the participating laboratories (10). The outcome of these parameters for our laboratory in 10 rounds (3rd to 12th) of proficiency testing for INH, RMP, EMB, and SM are shown in Table 4.

In total, 240 isolates were tested with the 25-well DST method within the framework of the WHO/IUATLD proficiency testing; the first six rounds each comprised 20 *M. tuberculosis* strains (10 strains in duplicate), and the last four rounds comprised 30 *M. tuberculosis* strains (10 strains in duplicate and 10 additional ones). The performance for INH testing was 100% for sensitivity, specificity, efficiency, reproducibility, and predictive value for resistance and susceptibility (Table 4). The performance of RMP testing for these characteristics varied from 96 to 100% because in two proficiency rounds one strain (in duplicate) was scored as susceptible, while the judicial result was resistant. In both cases, the MICs determined for these strains were 0.5 to 1 μg/ml, which is close to the breakpoint value of 1 μg/ml, marking the border between susceptible and resistant. The performance of SM and EMB testing ranged from 91 to 100% and 85 to 100%, respectively. For SM, all disagreements between our results and the judicial results relate to test strains with an intermediate level of resistance of 5 μg/ml, which cannot be reported as such in the WHO/IUATLD proficiency studies because either resistance or susceptibility should be scored for all strains. Similarly, for EMB, intermediate MICs were found for two strains. In addition, two strains were found false-resistant and one was false-susceptible for EMB compared to the juridical results. In rounds 3 and 5, we found a total of 10 strains with an EMB MIC of 10 μg/ml, which were reported susceptible at that time. Because of these disagreements found between our results and the juridical results in the proficiency testing for EMB, we adjusted our interpretation of this MIC and have since obtained better results. The 25-well DST method was 100% reproducible for all four drugs (Table 4).

Results of PZA susceptibility testing. From 1998 to 2005, 7,956 isolates of *M. tuberculosis* were tested for PZA susceptibility in our laboratory, but 930 (11.7%) of those isolates did not grow on the agar with PZA because of the lower pH required for PZA DST. In Table 3, the MIC distribution of PZA is shown for the remaining 7,026 isolates. It follows from these data that the majority of the isolates (87.2%) was inhibited by PZA concentrations of ≤20 μg/ml and 11.7% was inhibited by the breakpoint concentration of 50 μg/ml. Thirteen isolates (0.3%) showed reduced growth at a PZA concentration of 100 μg/ml and 55 isolates (0.8%) were resistant to 100 μg/ml PZA.

DISCUSSION

The high-throughput, 25-well DST method has proven to be very useful in our laboratory. In the past decade, more than 12,000 *M. tuberculosis* isolates from The Netherlands have been tested in this system within the framework of the national surveillance project (9, 22). The method is also suitable for DST of nontuberculous mycobacteria. DST on such isolates is performed in our laboratory only upon request, resulting in the testing of about 300 nontuberculous isolates yearly.

The quality of the 25-well DST method proves to be of a high standard. If the plates are kept for no longer than 3 weeks, the five control strains yield highly standardized MICs. In the second-line quality control, consisting of the exchange and retesting of mycobacterial isolates in a blind manner, the reproducibility of the 25-well DST method appears to be nearly 100%. In the proficiency studies of the WHO/IUATLD, the scores for the two most important first-line drugs, INH and RMP, are almost invariably 100%. Some problems are faced with the reading and interpretation of DST for SM and EMB. However, these problems mostly concern strains with exceptional MICs and the same problems with the interpretation of such MICs are faced when using other DST methods. Moreover, in the daily routine, strains with intermediate susceptibilities are reported as such, which is not possible in the proficiency study of the WHO.

One of the disadvantages of the current methods for DST of mycobacteria is that they are based on a phenotypic approach involving actual growth of the slowly growing mycobacteria as the indicator of resistance. Currently, for RMP-resistant strains and for some of the INH-resistant strains, reliable molecular tools are available. In most cases, the 7H10 agar DST method allows an indicative interpretation of the resistance after 6 days of incubation. This means that the speed of this method is almost comparable to those of the liquid medium DST methods, such as BACTEC and MGIT.

In many DST methods, problems are faced by testing susceptibility to PZA. This is mainly because the optimal activity

TABLE 4. Performance of the 25-well DST method in the WHO/IUATLD Supranational Reference Laboratory Network DST proficiency testing[a]

Anti-TB drug and round of testing	Sensitivity	Specificity	PV for:		Efficiency	Reproducibility
			Resistance	Susceptibility		
Streptomycin						
3	100	100	100	100	100	100
4	80	100	100	83	90	100
5	100	100	100	100	100	100
6	100	100	100	100	100	100
7	67	100	100	67	80	100
8	100	100	100	100	100	100
9	100	100	100	100	100	100
10	89	100	100	83	93	100
11	100	100	100	100	100	100
12	75	100	100	83	89	100
Avg	91	100	100	92	95	100
Isoniazid						
3	100	100	100	100	100	100
4	100	100	100	100	100	100
5	100	100	100	100	100	100
6	100	100	100	100	100	100
7	100	100	100	100	100	100
8	100	100	100	100	100	100
9	100	100	100	100	100	100
10	100	100	100	100	100	100
11	100	100	100	100	100	100
12	100	100	100	100	100	100
Avg	100	100	100	100	100	100
Rifampin						
3	80	100	100	83	90	100
4	100	100	100	100	100	100
5	100	100	100	100	100	100
6	100	100	100	100	100	100
7	100	100	100	100	100	100
8	75	100	100	86	90	100
9	100	100	100	100	100	100
10	100	100	100	100	100	100
11	100	100	100	100	100	100
12	100	100	100	100	100	100
Avg	96	100	100	97	98	100
Ethambutol						
3	67	100	100	88	90	100
4	100	92	88	100	95	100
5	33	100	100	50	60	100
6	100	100	100	100	100	100
7	75	100	100	86	90	100
8	100	80	83	100	90	100
9	93	100	100	93	97	100
10	83	100	100	94	95	100
11	100	100	100	100	100	100
12	100	100	100	100	100	100
Avg	85	97	97	91	92	100

[a] The results and averages of 10 rounds of proficiency testing are shown, involving in total 240 *M. tuberculosis* strains. PV, predictive value.

of PZA requires a lower pH, and a lower pH reduces mycobacterial growth (12, 18). In the early 1990s (also in our laboratory), the outcome of DST of PZA appeared to be rather unreliable because most strains failed to grow on the low-pH medium. However, further modification and standardization of the medium in 1997 significantly improved the test, resulting in reliable PZA susceptibility data for more than 85% of the isolates. Essential for this modification was keeping the pH of

the agar between narrow limits around pH 5.7, since at pH 5.5 a significant proportion of the isolates do not grow and at pH ≥6.0 the MICs for PZA sharply rise.

Most of the tuberculosis cases worldwide are found in Third World settings. Rising levels of resistance are especially recorded in the former USSR but also in parts of Asia (30). Because in most of these areas poor possibilities are available for the laboratory diagnosis of tuberculosis, the WHO has identified the expansion of culture and drug susceptibility testing capacity as one of the new challenges in the fight against TB (1). Resistance testing is either too expensive with the modern techniques or too complicated with the conventional Löwenstein-based approach. In these cases, the 25-well 7H10 agar DST method may be a suitable alternative. It offers the possibility to produce hundreds of plates a day and to distribute them to other centers for inoculation, incubation, and reading. These factors can facilitate therapy guidance and surveillance of resistance in areas where resistance testing is not in sight. In The Netherlands, the cost of material for one plate amounts to about €4. This includes true serial MIC testing for INH, RMP, SM, and EMB. Currently, the usefulness of this high-throughput DST method is being evaluated in high-tuberculosis-incidence settings in Indonesia, Hong Kong, Mongolia, Kiev, and various Latin American countries.

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