

## Rapid drug susceptibility testing of *Mycobacterium tuberculosis* using conventional solid media

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**ABSTRACT:** Radiometric methods for *M. tuberculosis* drug susceptibility testing yield much faster results than standard techniques; however, these methods require sophisticated equipment and are expensive. We investigated a rapid drug susceptibility testing method for isoniazid, rifampin, ethambutol, streptomycin and pyrazinamide in specimens from 197 patients with pulmonary tuberculosis using a simplified agar-dilution method.

Middlebrook 7H11 agar solid medium and microcolony detection were used to test sputum from 64 smear-positive, and from 70 culture-positive but smear-negative patients. Culture-positive material from bronchoscopy, surgical biopsy, pleural fluid or gastric fluid of 63 patients was tested.

In 64 smear-positive patients, the median time for final susceptibility results was 11 days (95% confidence interval (95% CI) 10–12 days) compared to 62 days (95% CI 56–66 days) with the standard method. In 133 smear-negative patients, results were available after a median of 35 days (95% CI 32–40 days) in contrast to 72 days (95% CI 62–83 days) with the standard method, regardless of whether or not sputum or other materials were used for primary culture. The rapid method detected all cases of single-drug resistance (n=20) and multidrug resistance (n=14) within 13 days (95% CI 9–17 days) in smear-positive patients (n=8), or within 38 days (95% CI 35–48 days) in smear-negative patients (n=26). Only one discrepancy was encountered in 985 resistance tests. Moreover, contamination was not observed.

Our rapid susceptibility testing method for *M. tuberculosis* on Middlebrook 7H11 agar is fast, practical and inexpensive. It provides an alternative when more sophisticated techniques are not available or affordable.

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Tuberculosis morbidity and mortality increased sharply in the 1980s [1]. Several factors contributed, including the human immunodeficiency virus infection (HIV), increasing poverty both in the developing and industrialized countries [1], and limited access to medical care. Besides the increased incidence of tuberculosis, the emergence of drug-resistant strains, especially in high-risk groups [2, 3], implies a considerable threat to public health [4]. Thus, it is extremely important to determine the susceptibility of individual *Mycobacterium tuberculosis* strains as rapidly as possible [5, 6]. In recent years, efforts have been made to shorten the time of susceptibility testing for *M. tuberculosis*. The radiometric culture system (BACTEC™) [7], which yields culture results much earlier than conventional procedures, is an example. Combining this method with susceptibility tests in drug-containing media shortens the time for final susceptibility results from 6–12 weeks to 2–4 weeks [8]. The method is technically advanced, but requires radioactivity, sophisticated equipment and laboratory expertise, and is expensive. Consequently, the method is not universally available and is not suitable for developing countries.

We established a modified agar-dilution drug susceptibility testing method using standard equipment and readily available solid media. In contrast to the World Health Organization (WHO) indirect standard methods [9–12] or the Centers for Disease Control (CDC) techniques [13, 14], which rely on pure cultures of *M. tuberculosis*, we started rapid susceptibility testing in smear-positive patients on the same day that the material arrived in the laboratory. The same rapid method was applied to smear-negative, culture-positive cases by using the first visible colonies in primary culture. Although the principles of this approach have been known for years [13, 15], no comparison between this rapid approach and the standard method has ever been made. We report the results of a prospective study using this approach in patients admitted to our hospital over a 16 month period.

### Material and methods

#### Patients

All 197 patients (133 males and 64 females) with culture-positive pulmonary tuberculosis hospitalized

between September 1993 and December 1994 were included in the study. Current standards for diagnostic procedures in tuberculosis were used [8, 16]. Clinical and demographic information was obtained from the patients hospital charts. Smear-positive cases showed a mean age of 39 yrs (26–84 yrs) and smear-negative cases of 41 yrs (1–87 yrs). The median of acid-fast bacteria observed in smear-positive cases during microscopy was 3+ bacteria-high power field<sup>-1</sup> (hpf). Only two patients suffered from a co-infection of tuberculosis and HIV.

### Specimens

Sputum testing was performed in 134 patients: 64 (48%) patients had acid-fast bacteria-positive smears, whilst 70 (52%) patients did not. Specimens obtained by fiberoptic bronchoscopy were used in 38 patients, by thoracocentesis of pleural effusion in 13 patients, by surgical biopsies in seven patients and by aspiration of gastric fluid in five patients. Specimens were examined by fluorescence microscopy, after staining for acid-fast bacteria using the auramine fluorescence method, and were classified as 4+, 3+, 2+, 1+ or 0 for acid-fast bacteria-hpf<sup>-1</sup> [13].

### Preparation of specimens for culture

Smear-positive specimens were centrifuged and the pellet was treated with N-acetyl-L-cysteine NaOH, as described previously [13]. Resuspension was carried out in 1 mL saline containing polymyxin B (200 U·mL<sup>-1</sup>), nalidixic acid (40 µg·mL<sup>-1</sup>), trimethoprim (20 µg·mL<sup>-1</sup>), azlocillin (100 µg·mL<sup>-1</sup>), and amphotericin B (10 µg·mL<sup>-1</sup>), to inhibit the growth of contaminants. To avoid large differences between the concentrations of inocula the suspensions were diluted in saline without antibiotics in patients with 4+ and 3+ smears (1:4 and 1:2, respectively), but were not diluted further in patients with 2+ and 1+ smears. Compared to the standard method, the inocula used in our method resulted in higher concentrations (approximately 10<sup>3</sup>–10<sup>4</sup> bacteria-inoculum<sup>-1</sup>) instead of 10<sup>2</sup> bacteria-inoculum<sup>-1</sup> as it is demanded by the standard method. This was done to avoid false susceptibility results using our new method.

For rapid susceptibility testing, a modified agar-dilution method was used [15]. In brief, 20 µL of the suspension was inoculated onto the central area of small Petri dishes (diameter 4 cm) containing Middlebrook 7H11 agar, 10% oleic acid dextrose catalase (OADC) and anti-tuberculous drugs. Middlebrook 7H11 agar, which is a 7H10 agar enriched with enzymatic digest of casein [17], stimulates growth, particularly of drug-resistant mycobacteria [13], more strongly.

In smear-negative patients, specimens were taken from the first colonies visible on solid medium, resuspended in saline, homogenized and adjusted to approximately McFarland 1.0 density with saline containing 0.1% Tween 80 (final volume 100–500 µL), and further diluted 1:50 in saline. This slightly higher concentration of the

mycobacterial suspension in the rapid test, as compared to the standard method (dilution 1:100), was again chosen to diminish false susceptibility results. Ten microlitres of the suspension, which corresponds to 10<sup>3</sup>–10<sup>4</sup> colony forming units (cfu), was inoculated onto Petri dishes with Middlebrook 7H11 agar supplemented as described above. For control purposes, the concentration of the mycobacterial suspension was always checked by one further dilution of 1:100 with saline.

### Culturing and identification

The plates were sealed with adhesive tape and incubated at 37°C. It had previously been found (data not shown from 70 simultaneously tested specimens) that CO<sub>2</sub> incubation allowed *M. tuberculosis* to be detected only 1 day earlier than when CO<sub>2</sub> was not employed. Thus, incubation was carried out without CO<sub>2</sub> to avoid the need for expensive CO<sub>2</sub> incubators. *M. tuberculosis* was identified by examining colony morphology [13, 18], using deoxyribonucleic acid (DNA)-amplification [19], and conventional biochemical identification tests [13] after subculture of all isolates.

### Breakpoints of susceptibility tests

Before application to clinical specimens, the rapid method was evaluated by testing 30 strains with known resistance and the breakpoints were adapted as described below. Since a complete correlation was found between the rapid method and the standard method, prospective testing of clinical specimens commenced in September 1993.

The following breakpoint concentrations of anti-tuberculous drugs were used by modifying the critical concentrations described by McCLATCHY [14] for isoniazid, 0.25 instead of 0.2 µg·mL<sup>-1</sup>, and ethambutol, 4.0 instead of 7.5 µg·mL<sup>-1</sup>. For isoniazid, the same concentration as with Löwenstein-Jensen medium was used, because extensive comparison between 0.25 and 0.2 µg·mL<sup>-1</sup> did not reveal any differences. For ethambutol, the lower critical concentration was used to avoid false susceptibility results. The critical concentrations for rifampin, 1.0 µg·mL<sup>-1</sup>, and streptomycin, 2.0 µg·mL<sup>-1</sup>, were not modified. Susceptibility results for pyrazinamide were obtained using Middlebrook 7H11 agar instead of Löwenstein-Jensen medium containing 0.5 µg·mL<sup>-1</sup> nicotinic acid amide (NSA) [20]. This approach was justified by the observation that, in 11 pyrazinamide-resistant and 320 pyrazinamide-sensitive *M. tuberculosis* strains, as well as in 25 *M. bovis* strains, no difference was observed between pyrazinamide sensitivity test results on Löwenstein-Jensen medium (pH 5.5) and the NSA method using either Löwenstein-Jensen medium or Middlebrook 7H11. To distinguish between *Mycobacterium bovis* and *M. tuberculosis*, Middlebrook 7H11 agar was supplemented with 1.0 µg·mL<sup>-1</sup> thiophen carboxylic acid (TCA). Strains were also inoculated on control plates without antibiotics.

Growth was examined daily, starting 4 days after inoculation, until visible growth of microcolonies was detected on the transparent control plates. Each visible colony was checked using a microscope (magnification  $\times 100$ ) [13, 21], since *M. tuberculosis* shows characteristic microcolonies which grow with serpentine cord shape, and appear irregular, dense and compact [18]. Thereafter, growth was checked every 3 days until the end of the third week. In all cases, no growth could be observed until 4 days after inoculation. All results were confirmed by the results obtained at the end of the third week of observation. Susceptibility was defined as complete inhibition at the described breakpoint concentrations for each antituberculous drug when microscopically confirmed growth was observed on the control plates [15, 22].

Simultaneously, at least once weekly, the minimal inhibitory concentration of a drug-susceptible strain of *M. tuberculosis* (H37Rv) was tested on separate plates as a quality control, with the following concentrations: isoniazid 0.03, 0.06, and 0.12  $\mu\text{g}\cdot\text{mL}^{-1}$ ; rifampin 0.25, 0.5 and 1.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ; ethambutol 1.0, 2.0, and 4.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ; streptomycin 1.0, 2.0, and 4.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ; and NSA 0.12, 0.25, and 0.5  $\mu\text{g}\cdot\text{mL}^{-1}$ .

In each case, the results of primary susceptibility testing were confirmed in parallel by a standard method after subculturing of all isolates. For this purpose, the indirect proportion method was performed using Löwenstein-Jensen solid medium as recommended by the WHO [9, 10], which is preferentially used in Europe and Germany [12]. Resistance was assumed if less than 99% inhibition of the original inoculate of *M. tuberculosis* was achieved on Löwenstein-Jensen medium containing the following concentrations of antibiotics: isoniazid (H) 0.25  $\mu\text{g}\cdot\text{mL}^{-1}$ ; rifampin (R) 32.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ; pyrazinamide (Z) 125.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ; ethambutol (E) 1.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ; streptomycin (S) 4.0  $\mu\text{g}\cdot\text{mL}^{-1}$  [12, 20].

#### Data analysis

Multiple continuous data were compared by nonparametric analysis of variance (ANOVA) (Kruskal-Wallis test) followed by multiple-comparison post-tests (Dunn's test). Direct comparison of two columns of continuous data was made with the nonparametric Mann-Whitney

U-test. Two-tailed p-values equal to or less than 0.01 were considered significant. Statistical analysis was carried out using a commercial statistics program (InStat, GraphPad<sup>®</sup>, San Diego, CA, USA).

#### Results

For the 64 patients with smear-positive tuberculosis, the final results of rapid drug susceptibility testing on Middlebrook 7H11 agar were available 6–19 days median 11 days; (95% confidence interval (95% CI) 10–12 days) following arrival of the specimens in the laboratory (table 1). In comparison, the conventional procedure required 62 days (95% CI 56–66 days), including primary culture of the specimens and subculture of *M. tuberculosis* colonies ( $p < 0.001$ ) (fig. 1).

Our method of rapid testing could also be applied to patients with smear-negative tuberculosis, when the first visible colonies of the primary cultures were used. In 70 sputum smear-negative cases in which a primary culture on solid media was performed, the final results of

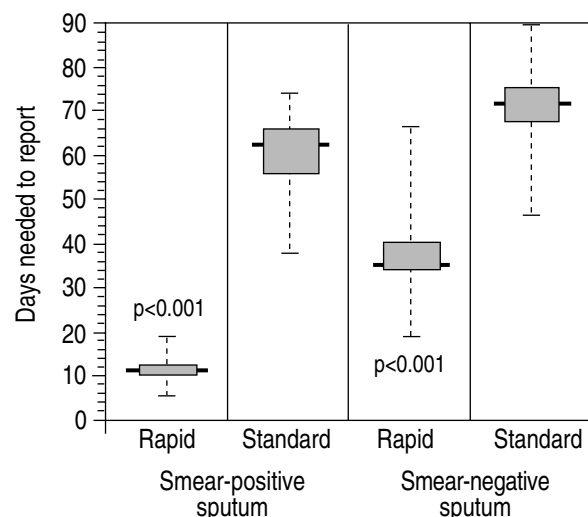


Fig. 1. – Days needed to report results of the susceptibility tests using the rapid technique in smear-positive cases (sputum;  $n=64$ ) and in smear-negative cases (sputum;  $n=70$ ) on Middlebrook 7H11 agar (rapid) compared to the standard method on Löwenstein-Jensen medium (standard). Values are expressed as medians (horizontal lines) with 95% confidence intervals (box) and range (vertical dotted line).

Table 1. – Results of rapid susceptibility testing using different specimens, in days needed to report results of the susceptibility tests

	Sputum smear-positive $n=64$		Sputum smear-negative $n=70$		Bronchoscopic specimens $n=38$		Pleural effusion $n=13$		Surgical biopsies $n=7$		Gastric fluid $n=5$	
	Rapid test	Standard test	Rapid test	Standard test	Rapid test	Standard test	Rapid test	Standard test	Rapid test	Standard test	Rapid test	Standard test
Mean days	11#	62	35#	72	34*	68	34*	74	35*	70	35*	73
95% CI days	10–12	56–66	33–40	67–75	31–46	63–82	29–41	59–85	29–44	58–90	30–45	65–88

95% CI: 95% confidence interval. \*: denotes  $p < 0.01$ ; and #: denotes  $p < 0.001$  versus the standard test.

drug susceptibility testing on Middlebrook 7H11 agar were obtained after a median of 35 days (95% CI 33–40 days) (including primary culture and the rapid test) compared to 72 days (95% CI 67–75) using the standard method on Löwenstein-Jensen medium ( $p < 0.001$ ) (fig. 1). In smear-negative cases, the median time required for the rapid susceptibility test alone was 9 days (95% CI 8–10, range 4–15).

In 63 patients, the rapid test was applied on specimens obtained by bronchoscopy, surgical biopsies, thoracentesis or gastric fluid suction. In these cases, we compared the time for susceptibility testing using both methods in relation to the type of specimen obtained from the patient. The median time for the final results of the rapid test was not different, regardless of which type of material was used for primary culture (table 1), and was significantly faster than the standard test ( $p < 0.01$ ).

The isolates of *M. tuberculosis* from 163 (83%) patients were susceptible against all five drugs tested. Resistance of *M. tuberculosis* strains to one or more of the standard drugs was detected in 34 patients (17%). In 20 patients, the microorganisms were resistant to a single drug and in 14 patients to two or more drugs. Altogether,

Table 2. – Resistance to antituberculosis drug in patients with culture-positive pulmonary tuberculosis as detected by the rapid and the standard method

Type of resistance	n	% of total (n=197)
Isoniazid	24	12
Streptomycin	21	11
Pyrazinamide	6	3
Rifampin	8	4
Ethambutol	5	3
Single drug resistance	20	10
Multidrug resistance	14	7

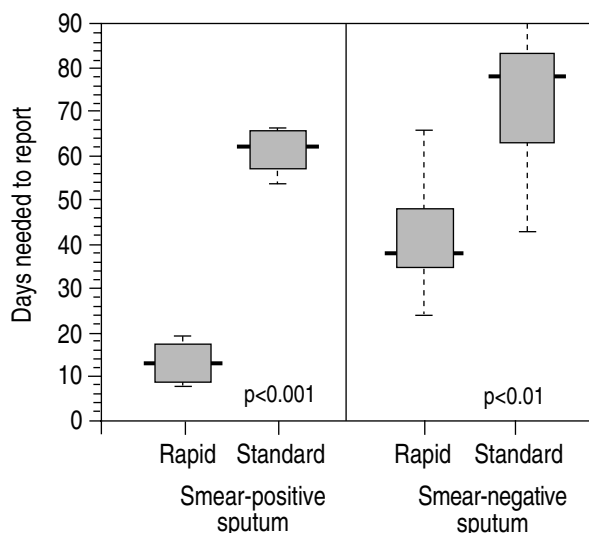


Fig. 2. – Days needed to report resistance of *M. tuberculosis* in 34 patients using the rapid technique on Middlebrook 7H11 agar (rapid) and the standard method on Löwenstein-Jensen medium (standard) in smear-positive and smear-negative cases. Values are expressed as medians (horizontal line) with 95% confidence intervals (box) and range (vertical dotted line).

64 incidences of resistance were detected (table 2). Resistance was demonstrated from sputum after a median time of 13 days in eight smear-positive patients and after a median time of 38 days (95% CI 35–48) in 26 smear-negative patients with the rapid method, compared to 72 days (95% CI 42–90) with the standard method ( $p < 0.01$ ) (fig. 2).

In each of the 197 *M. tuberculosis* strains, five drugs were tested using the rapid method. From these 985 tests, only one rapid susceptibility testing on Middlebrook 7H11 agar showed resistance against ethambutol, whilst the conventional testing showed susceptibility. In all other tests, the results of the rapid testing method were confirmed by those of standard susceptibility testing on Löwenstein-Jensen medium.

A further advantage of our method was that no contamination was observed.

## Discussion

A simple modification of the recommended procedure for direct susceptibility testing of *M. tuberculosis* was used to decrease the time necessary for susceptibility results without the need for special equipment or CO<sub>2</sub> incubation. In smear-positive cases, this approach required less than 2 weeks instead of 8–12 weeks with the standard solid media methods [8, 23, 24]. Furthermore, in smear-negative cases, this approach reduced the time needed for drug testing using different types of specimens by nearly half, from more than 10 weeks to 5 weeks when solid media were used for primary culture.

The notion that rapid susceptibility testing of *M. tuberculosis* had a lower reliability compared to the standard procedure could not be confirmed. On the contrary, in 197 patients 985 susceptibility tests for single drugs (five for each strain) were performed, of which only one was incorrect by showing resistance rather than susceptibility to ethambutol. In our opinion, the three most important measures to minimize the detection of false susceptible strains are: 1) providing an increased concentration of inoculum; 2) accepting only total inhibition for the definition of susceptibility; and 3) checking the colony morphology microscopically. Our method demonstrated a reliability similar to the standard method. However, we recommend a confirmation for any resistant strain by standard methods on Löwenstein-Jensen medium, Middlebrook 7H11 agar or by the BACTEC<sup>®</sup> technique until further studies have confirmed our results.

Direct susceptibility testing of *M. tuberculosis* from clinical specimens or from material obtained from primary culture is not a new approach and is basic in the standard manual for mycobacterial laboratories [13]. However, studies evaluating the rapid susceptibility testing of *M. tuberculosis* are rare. To our knowledge, only one other study has been performed [25]. The authors were able to report on susceptibility using a direct test on solid media within 3–4 weeks; however, no comparison of the standard method on solid media was made.

In terms of speed, every new method must be compared with the radiometric systems, the most important new culture system [7]. The BACTEC™ system employs a <sup>14</sup>C-labelled metabolic substrate, palmitic acid, which is metabolized to <sup>14</sup>CO<sub>2</sub> in the presence of viable mycobacteria. The amount of this radioactive metabolite can be quantified and used to detect mycobacterial growth within 1–2 weeks [25, 26]. By using media complemented with drugs, this method is also suitable for testing the susceptibility of isoniazid, rifampin, ethambutol, streptomycin and pyrazinamide within a mean of 2–3 weeks following the arrival of the specimens [24, 25].

However, the radiometric method has some important disadvantages. Firstly, the BACTEC™ method requires sophisticated and expensive equipment, which is not universally available. A recent survey revealed that less than 50% of US mycobacterial laboratories used a BACTEC™ system for detection, and only 25% for susceptibility testing of mycobacteria [24]. In developing countries, radiometric methods are available only in a few specialized centres. Moreover, the costs of a single test with the BACTEC™ system are relatively high, particularly in those places where a high frequency of resistance warrants an urgent need for susceptibility tests. Furthermore, direct susceptibility testing with the BACTEC™ system using liquid media and sophisticated methods, such as growth index detection by radiometric techniques, is much more difficult to standardize than the methods using solid media. Finally, non-radioactive methods present no problems with waste disposal.

The rapid drug susceptibility test on Middlebrook 7H11 agar described here avoids these limitations of the BACTEC™ system and provides susceptibility testing results within comparable time.

The early availability of susceptibility testing in smear-positive pulmonary tuberculosis has many advantages. Firstly, smear-positive cases are those with the highest risk of transmission. In addition to other methods of infection prevention our method might further help to control the spread of resistant organisms in high-risk patients. Secondly, the isolation time of hospitalized patients can be shortened, thereby reducing costs. Thirdly, the development of new types of resistance in patients infected with multidrug resistance during standard chemotherapy is a time-dependent process, which is very unlikely to occur within 14 days of inappropriate treatment [27].

Although the method of susceptibility testing described has been shown to be suitable for the investigation of nontuberculosis mycobacteria (NTM), patients with this type of infection were excluded in the study, since the susceptibility testing of NTM lacks appropriate standardized controls.

In summary, our method of rapidly testing *M. tuberculosis* susceptibility on Middlebrook 7H11 agar is fast, reliable and inexpensive. The method requires no additional equipment or material. The method can be integrated into the sequence of routine laboratory work in all laboratories around the world performing drug susceptibility testing on solid media.

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