



## Screening of the antimycobacterial activity of novel lipophilic agents by the modified broth based method



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### ABSTRACT

Most of the introduced susceptibility methods of *Mycobacterium tuberculosis* have some disadvantages for screening. Therefore, the selection of susceptibility assay for evaluating candidate agents must be determined case by case. In this study, we evaluated the validity of a modified broth dilution-based assay in comparison to the gold standard proportional method for microbial sensitivity test of new lipophilic compounds candidate as antitubercular agents. The *in-vitro* susceptibilities of 114 *M. tuberculosis* strains were separately tested against isoniazid and two lipophilic antitubercular agents (derivative of dihydropyridines) by employing the standard proportional method and a modified broth dilution-based assay. The results for isoniazid testing showed 100% concordance for sensitivity, specificity and reproducibility. In the case of microbial sensitivity test of lipophilic compounds, comparison of the results obtained from these two methods indicates a significant superiority of the modified method over the standard method. Considering the other advantages of this modified method, we concluded that this modified broth dilution-based assay could be utilized effectively for the susceptibility testing of new lipophilic compounds candidate as antitubercular agents.

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### 1. Introduction

Emergence of drug-resistant tuberculosis represents an increasingly major health problem worldwide. Globally in 2014, 480 thousand people developed multidrug-resistant tuberculosis (MDR-TB), and 123 thousand deaths from MDR-TB were detected and reported. Therefore, new, effective and well-tolerated treatments for tuberculosis are needed to end the global tuberculosis epidemic [1]. Over the past few decades, scientists have synthesized several new drugs, and derivatives of old drugs, in an attempt to find new treatments for tuberculosis [2,3]. In this way, one of the most important steps in the preclinical drug evaluation of new antitubercular agents is the susceptibility screening of *Mycobacterium tuberculosis* [4,5].

In the 1960s, Canetti et al., described the first standard drug susceptibility test method for *M. tuberculosis*, which performed on

Löwenstein–Jensen (L–J) medium with and without the drugs to be tested [6]. Although many laboratories still use this method, several alternative drug susceptibility testing (DST) methods have been introduced, each with advantages and disadvantages [7]. Using an egg-based L–J medium for DST requires heat for inspissations (80–85 °C for 45 min); therefore, it cannot evaluate heat-labile substances [8–10]. The other disadvantage of this method is some loss of drug activity as a result of binding to the egg proteins [10,11]. The agar-based method (Middlebrook 7H10 agar) has similar disadvantages, showing a reduction of drug activity as a result of binding to the agar [11]. Although the automated liquid medium DST methods (BACTEC 460TB, MGIT 960 system) eliminate these problems, they require an automated system that is expensive and not available in many less-equipped laboratories, which has inhibited their widespread implementation [7,12,13].

Imani Fooladi et al., by combining the two abovementioned methods (Middlebrook 7H9 broth, L–J medium), suggested a modified broth macro-dilution-based method for the drug susceptibility testing of *M. tuberculosis* against new heat-labile antitubercular agents. They only evaluated this method on *M. tuberculosis* strain H37Rv and a few other strains [8].

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The selection of a suitable screening assay for the microbial sensitivity testing of new antimycobacterial candidate drugs requires the consideration of several parameters including: sensitivity, expensiveness, radiometric disposal, need for high technology, high throughput, drug stability in culture mediums, potential to detect the precise minimum inhibitory concentration (MIC) as well as percent of growth inhibition, and rapidity. However, most of the introduced methods have some disadvantages for screening. Therefore, the selection of an antimycobacterial susceptibility assay for evaluating candidate drugs must be determined case by case.

While determining the inhibitory activities of some newly synthesized lipophilic antituberculosis drugs (derivative of dihydropyridines) against *M. tuberculosis*, [14] we initially carried out susceptibility testing by the proportional method on L–J medium. Surprisingly, no inhibition was observed. A review of the literature suggested that the inactivation of drug substances may occur due to interference with the L–J medium [10,11].

This study aimed to analyze the performance of a new modified broth-based method for the MIC-determining of lipophilic molecules, in comparison to the standard method on L–J medium. The susceptibilities of 114 *M. tuberculosis* strains were evaluated against isoniazid and two lipophilic antituberculosis drugs (derivative of dihydropyridines) employing both methods and the concordance between them was calculated.

## 2. Materials and methods

*In-vitro* mycobacterium susceptibility tests were carried out by two different methods: the proportional method on L–J medium and modified broth macro-dilution assay.

### 2.1. Bacterial strains and inoculums

*M. tuberculosis* H37Rv (American-type culture collection 27294) was the standard strain, and 113 isolates (pulmonary and extra pulmonary) from patients referred to the regional reference laboratory of tuberculosis in Mashhad, Iran were included in this study. All isolates were identified as *M. tuberculosis* by Ziehl–Neelsen staining, conventional biochemical and phenotyping methods and polymerase chain reaction (PCR). All isolates were stored in Middlebrook 7H9 broth, containing 10% glycerol at  $-20^{\circ}\text{C}$ . Substantially frozen stocks were subcultured on L–J mediums (Merck, Germany) and incubated for 4 weeks (cells were in the exponential phase of growth), and then bacterial suspensions corresponding to 1 McFarland turbidity were prepared in Middlebrook 7H9 broth from fresh colonies. Final concentrations of  $3 \times 10^7$  CFU/ml and  $3 \times 10^5$  CFU/ml of each isolate were prepared by adding Middlebrook 7H9 (inoculums  $10^{-1}$ ,  $10^{-3}$ ).

### 2.2. Drug preparation

Stock solutions of isoniazid (Sigma Chemical Co.) and two dihydropyridine derivatives were prepared – the isoniazid in deionized water and the lipophilic compounds in dimethyl sulfoxide (DMSO) – and sterilized by passage through a syringe filter (BIOFIL,  $0.22 \mu\text{m}$ ). The final concentrations in the L–J medium prior to testing were 0.2, 1, 2  $\mu\text{g/ml}$  for isoniazid, and 1, 2, 4, 8, 16, 32, 64  $\mu\text{g/ml}$  for the lipophilic compounds. To assure a statistically accurate comparison between the two methods, the inoculums, stock solutions and final concentrations in the 7H9 broth were the same as in the L–J medium. In this study, isoniazid was selected as the reference drug because many studies have shown that it is the best indicator for antitubercular susceptibility method evaluation, compared to other antitubercular drugs, and the difference in MICs detected by the broth and L–J medium was minimal for isoniazid [15].

### 2.3. Susceptibility test by standard method on L–J medium

Susceptibility testing against isoniazid and the lipophilic compounds was performed on L–J (Merck, Germany) medium, as described by Canetti et al. in the regional reference laboratory of tuberculosis, Mashhad, Iran. Briefly, equal amounts of two different dilutions (1:10 and 1:100) of a standardized inoculum (turbidity equal to the 1 McFarland standard) were inoculated onto L–J medium with and without the drugs to be tested. After 28 to 42 days incubation, resistance percentage for this drug was calculated by dividing the total number of colony-forming units (CFU) on the drug-containing medium to the total number of colonies growing on the drug-free medium. A 1% standard cut-off value was used for the interpretation of resistance. Therefore, a culture with a resistance rate of less than 1% was considered susceptible to that particular drug at that concentration, while a culture with a resistance rate greater than or equal to 1% was considered resistant to that particular drug [6].

### 2.4. Evaluation of anti-mycobacterial activity by modified broth dilution-based method

The modified method was performed as described by Imani Fooladi et al. [8]. In brief, Middlebrook 7H9 (FLUKA chemie) broth was prepared and enriched with 10% ADC (bovine albumin fraction V, dextrose, catalase), and Tween80 (0.05%, v/v).

For each strain, two sets of test tubes were prepared (A, B). Each set of test tubes consisted of: (a) test tubes contained 1000  $\mu\text{l}$  of the Middlebrook 7H9 broth, with serial dilutions of these two lipophilic compounds, which had been prepared freshly; (b) a drug-free control tube contained 1000  $\mu\text{l}$  of the Middlebrook 7H9 broth with no additive; (c) a solvent control tube, which the test medium was supplemented with DMSO at the highest concentration used in this study (4%, v/v); and (d) Middlebrook 7H9 broths containing 0.2, 1 and 2  $\mu\text{g/ml}$  of isoniazid. Then, 100  $\mu\text{l}$  of inoculums ( $10^{-1}$ ,  $10^{-3}$ ) was added to the tubes of each set (A, B), respectively.

After 7 days of incubation at  $37^{\circ}\text{C}$ , 100  $\mu\text{l}$  of each tube was inoculated on L–J medium (without any drug), and incubated at  $37^{\circ}\text{C}$  for 28 days. After 28 days, visible colonies were interpreted as bacterial growth; if there were no colonies, the L–J medium was inspected for further two weeks, and the results were reported after 42 days [6]. Colonies on each tube of the L–J medium were counted, and the number of colonies in the test tubes (transferred from drug-containing tubes) was compared with the number of colonies in the control tubes (transferred from drug-free tubes), and the percent of growth inhibition was calculated with the following formula:

Percent of inhibition

$$= (1 - (\text{colony count of test sample} / \text{colony count of drug - free control})) \times 100$$

The minimum bactericidal concentration (MBC) was defined as the lowest drug concentration that totally prevents colony formation, and minimum inhibitory concentration ( $\text{MIC}_{90}$ ) was defined as the lowest drug concentration that inhibits formation of more than 90% of colonies (reducing the bacterial load by 1 log unit), in comparison to the drug-free control.

Resistance was expressed as a growth of 1% or more of the bacterial population on media containing the breakpoint concentration of isoniazid; the breakpoint value was defined as 0.2  $\mu\text{g/ml}$  [9,13,16,17]. Data were analyzed using SPSS (version 16.0.0, 2007, SPSS Inc). Using Cohen's kappa coefficient, the agreement between the two methods was measured. Cohen's kappa coefficient is the most commonly used statistic to assess the degree of agreement

**Table 1**

Susceptibilities of *M. tuberculosis* strains against isoniazid at breakpoint concentration (0.2 µg/ml), determined by the proportional method on L–J and modified broth-based methods.

Strains	Method	
	Proportional method on L–J <sup>a</sup>	Modified broth based
H37Rv	The H37Rv was susceptible	The H37Rv was susceptible
77 susceptible strains	All 77 isolates were susceptible	All 77 isolates were susceptible
36 resistant strains	All 36 isolates were resistant	All 36 isolates were resistant

a: Lowenstein–Jensen, b: susceptible, c: resistant

between two tests. A calculated kappa equal to 1 indicates perfect agreement, whereas a kappa of 0 indicates agreement equivalent to chance [13,16,18]. P value < 0.05 was considered to be statistically significant. All antitubercular assays were performed in duplicate, and the mean values of inhibition were indicated.

### 3. Results

Initially, susceptibilities of all strains against isoniazid were measured by the proportional method on L–J medium. Seventy-seven isolates showed no growth, which was interpreted as susceptible (MBC ≤ 0.2 µg/ml), and 36 isolates showed more than 1% growth at concentration of 0.2 µg/ml, which was interpreted as resistant against isoniazid. The *M. tuberculosis* strain H37Rv also identified as susceptible. Then, the susceptibilities of all strains were tested against isoniazid by the modified broth-based method. All 77 susceptible strains, as well as the H37Rv strain, were totally inhibited by isoniazid at concentration of 0.2 µg/ml, and all resistance strains showed more than 1% growth (2–21% growth) by this method, which was interpreted as resistance (see Table 1).

The degree of concordance between two methods was evaluated by calculating the kappa value. The indicator kappa value was  $\kappa=1$ , which allows us to assert that there was a perfect concordance between both methods. There was 100% concordance between the modified broth-based method and the proportional method in the determination of MIC<sub>90</sub> and MBC of isoniazid for susceptible strains. In the case of resistant strains, there was also 100% concordance between the two methods in the detection of resistant strains, based on the breakpoint value (0.2 µg/ml); although, some MIC<sub>90</sub>s obtained by modified broth were one-fold dilution lower than MIC<sub>90</sub>s obtained with the standard method.

The susceptibilities of all strains against lipophilic compounds were measured by two methods. Using the standard method with L–J medium, no inhibitory activities were detected against all clinical isolates and the H37Rv strain, at all concentrations. In contrast, when using the modified broth-based method, all clinical isolates and the H37Rv strain showed significant levels of inhibition (11–100% for the susceptible group, and 0–98% for the resistant group) by different concentrations of lipophilic compounds. Inhibition percent of *M. tuberculosis* isolates and H37Rv at different concentrations of lipophilic compound by modified method are shown in Table 2.

To insure that the solvent had no effect on bacterial growth, a solvent control was prepared and the same inoculums used in the experiment were utilized; no inhibition was observed.

### 4. Discussion

Due to the interest of scientists in developing new antitubercular agents, it is necessary to have a screening assay to evaluate the mycobacterium inhibitory activity of new drugs, especially if lipophilic agents are to be tested, as they tend toward inactivation in L–J mediums [10]. Preferred susceptibility methods should be inexpensive, practical, use appropriate mediums for drug stability, be able to determine precise MIC as well as percent of growth inhibition, and provide reliable results compared to the standard method.

In this study, we calculated the sensitivity (ability to detect true resistance), specificity (ability to detect true susceptibility) and reproducibility (agreement between duplicate cultures) of the modified broth dilution method in comparison to the gold standard proportional L–J medium. The results for isoniazid testing against 113 isolates and the H37Rv strain showed 100% concordance for sensitivity, specificity and reproducibility. The calculated kappa coefficient was one ( $p=0.000$ ).

The facts that the difference between MICs detected by broth and L–J medium was minimal for isoniazid [15], involvement of a large sample size, and showed 100% concordance in susceptibility testing; suggested that the results of the modified broth-based method were completely confirmed by the L–J method and found to be a reliable method for susceptibility screening of *M. tuberculosis*. In addition, the lipophilic compounds tested in this study had no inhibitory activities against any strains of *M. tuberculosis* on the L–J medium; though, they showed different antitubercular activity (0–100%) with the modified broth-based method (see Table 2).

Considering the results obtained by this modified method and failure of standard method using L–J medium to detect inhibitory activities of the lipophilic compounds, it could be assumed that the cause of failure is due to interference of the L–J medium components with these drugs. Also it can be concluded that this modified broth based method is superior and supply an ideal environment for lipophilic compounds to do their antimycobacterial activity without interference.

Although antitubercular DSTs are carried out mainly using the standard proportional method on L–J medium and Middlebrook

**Table 2**

Comparison between inhibition of *M. tuberculosis* isolates at different concentrations of lipophilic compound by two methods.

Strains	Method	Range of inhibition						
		1 mg/mL	2 mg/mL	4 mg/mL	8 mg/mL	16 mg/mL	32 mg/mL	64 mg/mL
77 susceptible	L–J <sup>a</sup>	0 <sup>b</sup>	0	0	0	0	0	0
	Modified broth	49%	57.5%	64.5%	73%	79%	85%	93%
36 resistant	L–J	0	0	0	0	0	0	0
	Modified broth	24.5%	37%	51%	57.5%	63%	76%	83.5%

<sup>a</sup> Lowenstein–Jensen

<sup>b</sup> No inhibitions

7H10 agar, they have many disadvantages. In addition to the inactivation of heat-labile drugs [8,10], reduction of drug activity can occur because of (a) binding of the drug to the agar (agar-based mediums), or proteins and phosphate of egg (egg-based mediums) [10,11,19,20]; (b) prolonged incubation time, which potentially reduces the potency of certain antimicrobials; and (c) less contact of organisms with appropriate concentrations of antimycobacterial substances on solid media [8,9]. Although, the two lipophilic compounds (derivative of dihydropyridines) tested in this study have relative heat stability, the above-mentioned disadvantages of L–J medium (all or in part) might explain their inactivation in the standard method.

The cell wall of mycobacteria is rich in lipids [21,22]; therefore, most antitubercular agents are lipophilic [23–27], and screening of these compounds for antitubercular activities requires compatible methods. As such compounds have a major binding affinity to egg components; the inactivation of lipophilic compounds in an egg-based medium could have adverse consequences on microbial sensitivity testing. However, further investigations are needed to determine the precise mechanisms of inactivation of these lipophilic compounds in an L–J medium.

Recently, broth-based DSTs have been recommended for clinical and research tests by the US Food and Drug Administration (FDA) and the World Health Organization (WHO), using commercial broth-based systems (BACTEC 460TB, MGIT 960 system) [17, 28]. Although these methods are rapid and improve the reproducibility of MIC determination [29], their high cost and high technological needs inhibit their widespread implementation, especially in less-equipped laboratories [7,12,30].

Imani Fooladi et al. combined the two aforementioned methods (Middlebrook 7H9 broth, L–J medium) and suggested the modified broth dilution method for the DST of *M. tuberculosis* against heat-labile anti-TB drugs. They only evaluated this method on *M. tuberculosis* strain H37Rv and a few other strains [8]. Since the study size was small, it is necessary to evaluate this method in a larger population to come to a more accurate and acceptable conclusion. To our knowledge, a large-scale evaluation of this modified method has not been assayed. The present study utilized a considerable number of strains, which were tested to determine the validity of this modified method. The results showed that this modified method could ease susceptibility testing of *M. tuberculosis* against new lipophilic antitubercular agents in less-equipped laboratories, especially because it requires no high technology or high cost. However, there are some limitations, such as a 5–7 week waiting period for final results, a requirement for two different mediums and greater amounts of manipulation, which can be hazardous. This modified assay proved to be an ideal method for preclinical susceptibility testing and determination of the exact MBCs as well as percent of inhibition of new antitubercular agents, especially heat-labile or/and lipophilic compounds. Although, utilization of this modified method for the susceptibility testing of first-line anti-TB drugs (e.g. rifampicin, which experiences some inactivation in L–J medium) may inhibit partial inactivation of the drug by the culture medium; however, before this method can be performed for routine drug susceptibility, further investigations are needed.

The complete agreement between the results obtained from the modified broth-based method and the proportional L–J medium in the susceptibility testing of 114 strains of *M. tuberculosis* against isoniazid suggests that this modified method is reliable and has validity, compared with the gold standard L–J method.

Considering the failure of the standard method for the susceptibility testing of lipophilic antitubercular drugs, and the reliable results obtained using the modified broth-based method, we conclude that performance of the modified method can diminish the interference effect of an L–J medium on lipophilic antitubercular drugs. The modified broth-based method is sensitive, inexpensive,

non radiometric, and offers the potential for screening without high technology instrumentation. Therefore, this modified method could be recommended for the susceptibility screening of *M. tuberculosis* against not only heat-labile drugs as mentioned by Imani et al. [10] but also lipophilic compounds candidate as antitubercular agents.

### Conflict of interest

There is no conflict of interest related to the material in the manuscript.

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### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jctube.2016.01.001.

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