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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.

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°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 3×10^7 CFU/ml and 3×10^5 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 μm). The final concentrations in the L–J medium prior to testing were 0.2, 1, 2 μg/ml for isoniazid, and 1, 2, 4, 8, 16, 32, 64 μg/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 μl 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 μl 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 μg/ml of isoniazid. Then, 100 μl of inoculums (10^{-1}, 10^{-3}) was added to the tubes of each set (A, B), respectively.

After 7 days of incubation at 37°C, 100 μl of each tube was inoculated on L–J medium (without any drug), and incubated at 37 °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:

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

The minimum bactericidal concentration (MBC) was defined as the lowest drug concentration that totally prevents colony formation, and minimum inhibitory concentration (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 μg/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