Evaluation of the microscopic observation drug susceptibility assay for detection of *Mycobacterium tuberculosis* resistance to pyrazinamide

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Abstract

The microscopic observation drug susceptibility assay (MODS) was evaluated to determine susceptibility to pyrazinamide in *Mycobacterium tuberculosis*, and compared with the broth microdilution method (BMM), absolute concentration method (ACM), and pyrazinamidase (PZase) determination. We tested 34 *M. tuberculosis* clinical isolates (24 sensitive and eight resistant to pyrazinamide) and the control strains *M. tuberculosis* H37Rv (ATCC 27294) and *Mycobacterium bovis* AN5. The MODS, BMM, ACM and PZase determination provided results in average times of 6, 18, 28 and 7 days, respectively. All methods showed excellent sensitivity and specificity ($p < 0.05$). Of the methods studied, the MODS proved to be faster, efficient, inexpensive, and easy to perform. However, additional studies evaluating the MODS in differentiating pyrazinamide-resistant and pyrazinamide-susceptible *M. tuberculosis* must be conducted with a larger number of clinical isolates.

Keywords: Microscopic observation drug susceptibility assay (MODS), pyrazinamide, resistance, susceptibility testing, tuberculosis
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Introduction

Tuberculosis (TB) is a worldwide health problem. It is estimated that 9.27 million cases occurred in 2007, and approximately 15% of these involved co-infection with human immunodeficiency virus [1]. Pyrazinamide is an important drug that is used in combination with isoniazid, rifampicin and ethambutol in the first-line treatment of TB [2]. Pyrazinamide needs to be converted to pyrazinoic acid by pyrazinamidase (PZase), which is effective against a population of semidormant *Mycobacterium tuberculosis* in the acid environment inside macrophages that cannot be reached *in vivo* by other drugs [3].

Pyrazinamide susceptibility testing *in vitro* is somewhat difficult to perform, because the acid pH of the medium required to maintain drug activity inhibits bacterial growth [4]. Conventional pyrazinamide susceptibility testing by the Lowenstein–Jensen (L-J) proportion method is most often used for *M. tuberculosis*, but this technique requires a minimum of 3–4 weeks to produce results [5,6]. Furthermore, we need to consider the fact that when the drug is included in the L-J medium and exposed to high temperature (85°C) during the preparation of the medium, its potency may be reduced [7].

New methods have been proposed recently for *M. tuberculosis* susceptibility testing, including the resazurin microtitre assay plate [8], the microplate Alamar Blue assay [9], the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide assay [10], the broth microdilution method (BMM) [11], and the microscopic observation drug susceptibility assay (MODS) [12,13]. These methods have the advantage of using a liquid medium, which can provide results in a shorter time.

The MODS [12] is fast and easy to perform. It also has a low cost and allows simultaneous detection of the bacilli and

the incorporation of drugs for the susceptibility testing. The method is based on the principle that *M. tuberculosis* grows faster in a liquid medium, and its characteristic growth 'cording formation' can be observed through an inverted light microscope [12–15]. Several studies have used the MODS to determine susceptibility to isoniazid and rifampicin [12,13,15–17], and it seems to be reliable as compared with the reference method. However, it has not been applied to detect susceptibility to pyrazinamide.

The aim of our study was to evaluate the application of the MODS for the determination of susceptibility to pyrazinamide in *M. tuberculosis*, and to compare it with the BMM, the absolute concentration method (ACM), and determination of PZase activity.

Materials and Methods

Mycobacterial strains

The pyrazinamide-susceptible strain *M. tuberculosis* H37Rv (ATCC 27294) and the pyrazinamide-resistant strain *Mycobacterium bovis* AN5 were used as controls in the susceptibility test for pyrazinamide. *Mycobacterium avium* (ATCC 13950) and *M. bovis* AN5 were used as positive and negative controls, respectively, for determination of PZase activity.

A total of 32 *M. tuberculosis* clinical isolates were selected from the mycobacteria collection of the Clinical Bacteriology Laboratory in the Clinical Analysis and Biomedicine Department of Maringá State University. Of these isolates, 24 were susceptible and eight were resistant to pyrazinamide, as assessed by the proportion method in L-J medium (pH 5.5) with a pyrazinamide concentration of 100 mg/L as a cut-off for resistance [18]. Automated DNA sequencing of a 1200-bp segment including the entire *pncA* open reading frame, as well as its regulatory region, was previously carried out [19] in pyrazinamide-resistant *M. tuberculosis* isolates.

Pyrazinamide solution

A stock solution of 20 000 mg/L pyrazinamide (Sigma, St Louis, MO, USA) in distilled water was prepared, filter-sterilized, and stored at −20°C until use. At the time of use, the stock solution was diluted to give the MICs for the MODS, BMM, and ACM.

MODS

The MODS was performed as previously described [16] in 24-well sterile plates (TPP, Trasadingen, Switzerland), in triplicate. Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA) supplemented with OADC Enrichment (BBL/Becton-Dickinson, Sparks, MD, USA) was prepared as previously described [12], and the pH was adjusted to 6.0 [19]. One millilitre of drug-free medium was added to each well, and the pyrazinamide stock solution was diluted to obtain final concentrations of 6.25–3200 mg/L. One hundred microlitres of mycobacterial inoculum, previously standardized according to 1 McFarland turbidity and diluted to 10^{-3} [16], was inoculated into each well of drug-containing medium, and also into the wells of the mycobacterial control. Each plate was covered with its lid and sealed along its edge with polyethylene tape. The plates were placed in a plastic bag to prevent evaporation, and incubated at 35°C in normal atmosphere. Mycobacterial growth was observed daily with an inverted light microscope at ×40 magnification (Olympus, CK 40) from the 3rd to the 15th day of incubation [13,16]. Mycobacterial growth was defined as the emergence of visually characteristic serpentine growth. The MIC results were interpreted on the day when distinct growth could be observed in the control wells for each *M. tuberculosis* isolate. The isolates that showed MICs ≤100 mg/L were considered to be susceptible to pyrazinamide [19].

BMM

The BMM was performed as previously described [11], in triplicate. First, 200 μL of distilled water was added to the outer wells of the 96-well microplates (Kartell, Milan, Italy). One hundred microlitres of Middlebrook 7H9 medium (Difco Laboratories), supplemented with OADC Enrichment (BBL/Becton-Dickinson) and with the pH adjusted to 6.0 [19], was added to each well. The pyrazinamide stock solution was diluted to obtain concentrations from 12.5 to 3200 mg/L. Five microlitres of bacterial inoculum standardized to 0.5 McFarland turbidity and further diluted to 10^{-2} [11] was inoculated into the wells of drug-containing media and also into the control wells. Each plate was covered with its lid and sealed along its edge with polyethylene tape. The plates were placed in a plastic bag to prevent evaporation, and incubated at 35°C in a normal atmosphere. The readings were made after 14, 20 and 28 days of incubation through visual observation of the growth. The MIC was defined as the lowest pyrazinamide concentration that exhibited no growth by visual reading, and the isolates were considered to be susceptible to pyrazinamide if their MICs were ≤100 mg/L [19].

ACM

The ACM was performed in triplicate in L-J medium (Difco Laboratories). The L-J medium was prepared according to the manufacturer's instructions, and the pH was adjusted to 5.2 with sterile 5 M hydrochloric acid (Quimex, São Paulo, Brazil) [5]. The pyrazinamide stock solution was diluted to