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Full Length Article

Rapid drug-susceptibility testing of *Mycobacterium tuberculosis* clinical isolates to first-line antitubercular drugs by nitrate reductase assay: A comparison with proportion method

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ABSTRACT

Objective/background: Early initiation of therapy in patients with tuberculosis is imperative for its control. Conventional methods of susceptibility testing such as the proportion method (PM) require visual detection and counting of colonies that takes up to 6 weeks. Rapid and simple phenotypic methods that have been endorsed by the World Health Organization can serve as alternatives.

Methods: In this study, we evaluated the colorimetric nitrate reductase assay, which utilizes the detection of nitrate reduction as an indicator of growth much earlier compared with PM (within 7–14 days). The susceptibility of 75 clinical isolates of *Mycobacterium tuberculosis* to four first-line antitubercular drugs was tested by nitrate reductase assay and compared with the standard PM. In this assay, inoculation was done on both drug-free and drug-containing Löwenstein–Jensen medium containing sodium nitrate. After incubation for 7–14 days, reduction to nitrite was taken as an indicator of growth, which was detected by color change on addition of Griess reagent.

Results: Agreement between nitrate reductase assay and PM was 100% for rifampicin, 97.30% for isoniazid, 93.30% for streptomycin, and 98.60% for ethambutol. Cost/isolate with this assay was found to be approximately two times lesser than that of PM. All results were obtained in 7–14 days by nitrate reductase assay, which was significantly rapid compared with 42 days taken for obtaining results by PM.

Conclusion: Nitrate reductase assay can be used as a rapid and inexpensive method for drug-susceptibility testing of *M. tuberculosis* for first-line antitubercular drugs without compromising accuracy of standard methods.

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Introduction

During the past two decades, the world has witnessed a dramatic increase in the incidence of tuberculosis (TB), particularly emergence of multidrug-resistant (MDR) strains that pose a major threat to the TB control program. In 2014, estimates indicated 480,000 new cases of MDR-TB worldwide and approximately 190,000 deaths from MDR-TB. More than half of these patients were in India, China, and the Russian Federation [1]. Factors contributing to the recent outbreak and continued spread of MDR-TB include upsurgence of human immunodeficiency virus infection, insufficient control procedures, and laboratory delays in identification and susceptibility testing of *Mycobacterium tuberculosis* isolates [2,3]. This emphasizes the need for rapid and cost-effective susceptibility testing to first-line antitubercular drugs to diagnose and treat MDR cases at the earliest. More than treating individual cases, it will ensure rapid control of spread of MDR epidemic.

The globally accepted standard methods of drug-susceptibility testing (DST) are the proportion method (PM), the absolute concentration method, and the resistance ratio method. These methods are based on visual detection of slow-growing colonies of *M. tuberculosis* and can take up to 6 weeks that may be crucial for early initiation of intensive-phase therapy and reduction of bacterial load in smear-positive cases [4]. Liquid medium-based automated culture systems such as the BACTEC 460 TB system [5], the mycobacterial growth indicator tube MGIT 960 [6], BacT/ALERT 3D [7], or ESP culture system II [8] require expensive substrate and equipment and are therefore not feasible in most developing countries [9]. Molecular tools such as line probe assays and Xpert MTB/RIF besides being expensive require expertise and may not differentiate active infection by picking DNA from even dead organisms [10]. Microscopic observation DST, although rapid and cheap, requires detailed staff training [11–14]. Colorimetric liquid medium-based susceptibility tests such as resazurin microtiter assay [15] and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [16] carry a biohazard risk through aerosol formation. Therefore, there is an urgent need for a test that is inexpensive, rapid, safer, and simpler to perform yet not compromising the accuracy of standard procedures. Nitrate reductase assay on solid medium is a susceptibility test well suited for this purpose. It is a noncommercial colorimetric assay where visual detection of color change on addition of Griess reagent [17] makes the test easy to interpret.

This study was aimed at comparing the indirect nitrate reductase assay (INRA) with the indirect proportion method (IPM) in terms of speed, cost, ease of performance, and accuracy for DST to first-line antitubercular drugs.

Materials and methods

The study was conducted over a period of 18 months from October 15, 2013, to April 15, 2015, in the Department of Microbiology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar, Kashmir, India. The study was approved by the Institute's Ethical Committee.

Strains

Seventy-five isolates of *M. tuberculosis* obtained from various clinical samples (sample distribution: 49 sputum, 8 bronchoalveolar lavage, 6 pus, 5 urine, 2 ascitic fluid, 2 pleural fluid, 1 cervical node aspiration fluid, 1 tracheal aspiration fluid, and 1 cerebrospinal fluid) were included in the study. The samples after decontamination were inoculated on standard Löwenstein–Jensen (LJ) medium and incubated at 37 °C for 6 weeks. All the isolates obtained thus were identified by standard biochemical tests [18]. DST was performed using IPM and INRA on fresh (3–4-week old) growths only.

Indirect proportion method

DST by IPM [18] on LJ media was performed at the following final drug concentrations: isoniazid (INH) at 0.2 μg/mL, rifampicin (RIF) at 40.0 μg/mL, streptomycin (STM) at 4.0 μg/mL, and ethambutol (EMB) at 2.0 μg/mL. In brief, two appropriate dilutions of the bacilli, 10^{-2} and 10^{-4} dilutions (undiluted = 10^{6}–10^{8} colony-forming units/mL), were inoculated on drug-containing and drug-free media, to obtain countable colonies on both media. One set of media bottles for testing one culture consisted of five LJ slopes (1 for neat, 2 for 10^{-2}, and 2 for 10^{-4}) and eight LJ drug-containing slopes (2 each for drugs INH, RIF, EMB, and STM, i.e., 1 each for 10^{-2} and 10^{-4} suspensions). Thus, a total of 13 LJ slopes were required. Slopes were put in a stand at a very slight angle from the horizontal plane and placed in an incubator at 37 °C.

The reference strain $H_{37}Rv$, which is susceptible to all standard anti-TB drugs, was used as susceptible control in each batch of tests.

The first reading of observable growth was taken on the 28th day and final reading was taken on the 42nd day. The colonies were counted only on the slopes seeded with the lowest inoculum that produced growth. The average number of colonies obtained for the two control slopes indicated the number of culturable particles contained in the inoculum. The average number of colonies obtained for the drug-containing slopes indicated the number of resistant bacilli contained in the inoculum. The percentage resistance was calculated as the ratio of the number of colonies on the drug-containing media to those on the control medium. If $R \geqslant 1\%$, the isolate was taken as resistant.

If the result of the reading made on the 28th day was "resistant," no further reading of the test for that drug was required: the strain was classified as resistant. If the result at the 28th day was "sensitive," a second reading was made on the 42nd day: this provided the definitive result.

Indirect nitrate reductase assay

DST by the INRA [19] was performed on standard LJ media at the final drug concentrations as mentioned in the "Indirect Proportion Method" section: sodium nitrate in critical concentration of 1000 μg/mL was incorporated into all drug-free and drug-containing media used for the assay. In brief,

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