International Journal of Mycobacteriology 5 (2016) 469–474

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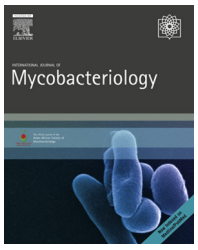

Full Length Article

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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ARTICLE INFO

Article history:
Received 27 May 2016
Accepted 8 June 2016
Available online 25 June 2016

Keywords:
Drug-susceptibility testing
Indirect nitrate reductase assay
Indirect proportion method
Mycobacterium tuberculosis

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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Peer review under responsibility of Asian African Society for Mycobacteriology.
http://dx.doi.org/10.1016/j.ijmyco.2016.06.006

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,

two-third loopful of fresh bacterial growth was passed in 2.5 mL of 7H9 broth taken in a sterile Bijou bottle with six glass beads. It was vortexed for 1 min and allowed to stand for 5 min. The supernatant was then transferred to a new sterile vial and left to sediment for 15 min. The turbidity was set at 1 McFarland using 0.067 M phosphate buffer (pH 6.8) and labeled as 'neat'. A 1:10 dilution was prepared from neat and labeled as 'S2'. Three drug-free tubes were inoculated with 0.2 mL of S2 and four drug-containing tubes with 0.2 mL of neat. All tubes were initially incubated at 37 °C for up to 7 days.

Reading of tubes

On the 7th day of incubation, Griess reagent was prepared shortly before use by mixing one part of 50% (v/v) concentrated HCl with two parts of 0.2% (w/v) sulfanilamide and two parts of 0.1% (w/v) *n*-1-naphthylethylenediamine dihydrochloride; 0.5 mL of this reagent was added to one drug-free medium of all the strains and observed for color change. Color change to pink magenta was suggestive of *M. tuberculosis* growth. This was followed by testing of four drug-containing media of that particular strain.

Interpretation

An isolate was considered sensitive if there was no color change in drug-containing tubes (Figs. 1 and 2) or the color was lighter than the drug-free control (Fig. 3); however, they were considered resistant if the color intensity in the drug-containing media was the same or more compared with that in the drug-free control (Fig. 4).

In the case of a negative result on a drug-free tube on the 7th day, the aforementioned procedure was repeated on the 10th and 14th days of incubation.

Chi-square test was used to detect significance and kappa test was used to analyze the agreement between the two tests.

Fig. 2 – Indirect nitrate reductase assay of test strain showing no color change in drug-containing media; sensitive to all the four drugs.

Fig. 3 – Indirect nitrate reductase assay of test strain showing less-intense color change in streptomycin and isoniazid as compared with drug-free control; sensitive to all the four drugs.

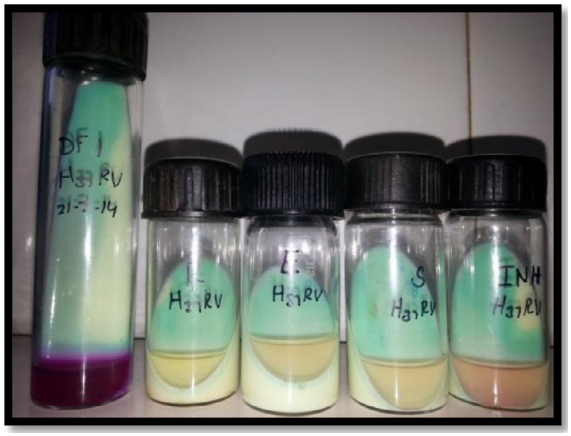

Fig. 1 – Indirect nitrate reductase assay of sensitive control; $H_{37}Rv$ showing no color change in drug-containing media.

Fig. 4 – Indirect nitrate reductase assay of test strain showing similar color change in ethambutol as compared with drug-free control; resistant to ethambutol.

Results

All the results obtained by INRA in our study were compared with the results of IPM in terms of ease of performance, cost/isolate, time to positivity, sensitivity, specificity, and accuracy (Table 1).

Although the same media and equipment were required for both the procedures, the amount of media, number of bottles, dilutions of inoculums, persons required, time to inoculate media, and time to incubate were significantly less in INRA. In addition, the inoculation of media using a loop in IPM was technically more demanding compared with simpler inoculation using pipettes in INRA. In our study, it took us just 40 s to inoculate one strain in INRA compared with 2–3 min of loop inoculation in IPM.

The cost/strain by INRA amounted to 14 INR (0.219 USD) and by IPM it was 36 INR (0.56 USD). This excluded the cost of the equipment already present in our laboratory; also the manpower utilized in this study was not included in the cost calculation for each strain.

Of the 75 isolates that were subjected to INRA during the study period, 27 (36%) isolates gave positive results on the 7th day, 30 (40%) isolates on the 10th day, and 18 (24%) isolates on the 14th day of incubation.

Of the 75 isolates, 70 tested sensitive and five tested resistant to RIF by IPM. The results of INRA for RIF were similar to those of IPM in all the isolates. Thus, a perfect agreement with the gold-standard PM was observed for RIF.

For INH, one isolate, identified as being sensitive by the PM, was falsely identified as resistant by INRA and one isolate resistant by the PM was falsely identified as sensitive by INRA. Thus, INRA was found to be 97.30% accurate in comparison with PM.

For STM, there were two false-positive and three false-negative results. An accuracy of 93.30% was observed with reference to PM.

For EMB, one of the seven isolates tested resistant by PM was falsely identified as sensitive by INRA. However, no false-positive result was found for EMB with INRA. Thus, an accuracy of 98.6% reflected a strong association of the two diagnostic methods for EMB.

Discussion

In this study, for INRA, 76% of the isolates showed results by 10 days compared with 42 days required by PM, indicating that the INRA is quite rapid. Our results were similar to the results of various studies [20–25] where fresh 3–4-week-old colonies were used for testing, as older cultures may result in unreliable susceptibility test results.

Cost is an important factor in resource-constraint areas of the world where most of the disease is prevalent. A cost/isolate of 14 INR (0.22 USD) by INRA as compared with 36 INR (0.56 USD) by IPM and much higher costs required for commercial and molecular assays emphasizes the need of this simple colorimetric assay to be routinely used in laboratories especially in low-income countries. The additional equipment, media, and infrastructure required for INRA are routinely present in most laboratories.

DST results of INRA to four first-line antitubercular drugs in our study gave favorable results for INH and RIF, the two most important first-line drugs for TB.

In our study, RIF had perfect agreement between the two methods. This observation was concordant with many previous studies [22,24,26–30]. As RIF is the most important drug in the treatment of TB and considered a surrogate marker for detecting MDR-TB, the results obtained in our study further validate the use of INRA as a routine method for screening of MDR-TB.

A slightly low sensitivity of INH was seen in our study, which was concordant with the studies conducted by Montoro et al. [22], Martin et al. [23], Mengatto et al. [24], Lemus et al. [26], Kumar et al. [30], and Poojary et al. [31].

The lower sensitivities to STM and EMB observed in our study have also been reported by other authors [9,32,33]. This might be overcome by adjusting the critical drug concentrations used in the INRA test, although it is well-known that STM and EMB are difficult drugs to test even by conventional methods.

For treating TB, time to positivity of various tests is critical. In our study, we observed that INRA is rapid compared with IPM but both need an additional time of 3–4 weeks for growing isolates from smear-positive clinical samples. This

Table 1 – Sensitivity, specificity, PPV, NPV, and accuracy of INRA as compared with IPM.

Antitubercular drug	IPM	INRA		Statistical analysis					
		Sensitive	Resistant	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	K value
Rifampicin	Sensitive	70	0	100	100	100	100	100	1
	Resistant	0	5						
Isoniazid	Sensitive	63	1	90.90	98.40	90.9	98.4	97.30	0.893
	Resistant	1	10						
Streptomycin	Sensitive	56	2	82.30	96.50	87.5	94.9	93.30	0.806
	Resistant	3	14						
Ethambutol	Sensitive	68	0	85.70	100	100	98.5	98.60	0.916
	Resistant	1	6						

INRA = indirect nitrate reductase assay; IPM = indirect proportion method; NPV = negative predictive value; PPV = positive predictive value.

time of 3–4 weeks needed for obtaining visible growth on media can be eliminated by applying the nitrate reductase assay directly on the clinical specimen. The time lag of 3–4 weeks between direct and indirect NRA is crucial for early initiation of therapy for TB, especially in areas with high prevalence of MDR cases.

Conclusions

In conclusion, INRA was found to be rapid and inexpensive; besides, it was easy to perform and interpret compared with the gold-standard IPM. It could act as an effective alternative to time-consuming or expensive methods of susceptibility testing especially in resource-constraint settings carrying maximum burden of TB.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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