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Research Article

Drug Susceptibility Testing of *Mycobacterium Tuberculosis* for Isoniazid and Rifampicin by Absolute Concentration and MTT method – A Comparative Study

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A B S T R A C T

Introduction: Tuberculosis has been a main cause of mortality and morbidity globally. In spite of having anti-tuberculous drugs, elimination of tuberculosis is difficult because of the drug resistance. In order to achieve vital therapy selection and prevention of resistance, expeditious detection along with drug susceptibility techniques are essential needs of the hour.

Aim: To evaluate the MTT assay and the absolute concentration method for determining *Mycobacterium tuberculosis* susceptibility to rifampicin and isoniazid

Methods: 71 isolates of *Mycobacterium tuberculosis* were tested for susceptibility to the first-line medications isoniazid and rifampicin using the MTT assay and the absolute concentration method after sputum samples that tested positive for acid-fast bacilli were gathered.

Results: Of the 71 isolates, 14% (10) were resistant to isoniazid and 9% (6) were resistant to rifampicin by absolute concentration method. Isoniazid resistance was seen in 13% (9) and 10% (7) were resistant to rifampicin by MTT assay. Isoniazid mono resistance was observed in 5 isolates and 1 isolate to rifampicin alone by absolute concentration method. Two isolates that were mono-resistant to rifampicin and four isolates that were mono-resistant to isoniazid were acquired using the MTT assay. Both the absolute concentration approach and the MTT experiment revealed that 7% (5) of the isolates were multidrug resistant.

Conclusion: Increasing numbers of resistant and multidrug-resistant tuberculosis (MDR-TB) strains have led to a daunting need for rapid, reliable, and accurate drug susceptibility testing. Hence, we concluded that the absolute concentration method is better than the MTT assay in terms of simplicity of performance though the results may be delayed.

Keywords: MTT Assay, Drug Susceptibility, MDR-TB, Absolute Concentration Method

Introduction

Tuberculosis still remains one of the major causes of mortality and morbidity globally.[1] The first anti-tuberculous drug was introduced five decades ago, but the elimination of tuberculosis is still like dreaming with open eyes.[2] The major reason for this is the development of drug resistance, which has been increasingly reported in both developed and developing countries.[3] The incidence of tuberculosis in the community first decreased with the introduction of anti-tuberculous medications in the late 1940s and early 1950s. Combining four medications—isoniazid (INH), rifampicin (RMP), streptomycin, and ethambutol—is an effective way to treat tuberculosis.[4] Streptomycin was the first anti-tubercular medication to be found.[5]

Multidrug-resistant Tuberculosis (MDR-TB) was reported for the first time in 1990. The bacterium exhibited resistance to the two most potent first-line anti-tuberculous medications, INH and RMP.[6] *Mycobacterium tuberculosis* strains that were resistant to both the first-line medications INH and RMP as well as to at least three of the six classes of second-line anti-tuberculous drugs (aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, and para-aminosalicylic acid) were identified as extensively drug-resistant tuberculosis (XDR) later in 2006.[7] In 2007, strains of the organisms that are completely resistant to all anti-tuberculous medications began to appear.[8] India is one of the countries with the highest number of MDR-TB cases. The other countries with top rankings are China, the Russian Federation, South Africa, and Bangladesh.[9] There were 58 countries where extensively drug-resistant TB (XDR-TB) has been reported.[10]

The advent of MDR-TB is the most concerning and upsetting issue in the pandemic of antibiotic resistance because patients who do not receive treatment are more likely to die.[11] The WHO coined the term MDR "hotspot" for the first time in history to describe areas where high MDR prevalence was noted. Places in nations or regions where the cumulative prevalence of MDR-TB has increased by 5% are referred to be "hotspots".[12] Analysing the WHO data from 216 countries and territories, 198 reported data of 558,000 patients collected in 2017 showed an average of MDR-TB in 82% of all cases of TB[13] where the major burden was from India.

The necessity of quick isolate identification and susceptibility testing in order to choose the best course of action to stop the spread of resistant organisms. But, due to the slow growth of M. tuberculosis, drug susceptibility testing of this organism has proved to be a challenge.[14] Therefore, we sought to evaluate the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and the absolute concentration method for identifying *Mycobacterium tuberculosis* susceptibility to INH and RMP.

Materials and Methods

Following institutional ethical clearance (IRB 09/130), the prospective study was carried out in the Department of Microbiology, Shri Sathya Sai Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Chennai Campus for a year, from April 2018 to March 2019. Outpatients' sputum samples were examined for acid-fast bacilli. A total of 105 samples were collected, out of which 71 were selected for the study. The remaining 34 samples were either contaminated or showed no bacterial growth. Samples positive for acid-fast bacilli were subjected to standard methods of biochemical tests for isolation and speciation of M. tuberculosis. All processing work was done in a Class II Biological Safety Cabinet as per recommendations. Informed consent were obtained from the patients. Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS) software.

Drug Susceptibility Testing

All the isolates grown were first speciated as M. tuberculosis and then subjected to susceptibility testing of 2 first-line drugs namely isoniazid and rifampicin by both absolute concentration method and MTT assay. M. tuberculosis H37Rv, the reference strain was used for the standardisation of drugs.

Absolute Concentration Method[15]

After placing the isolates in a test tube, 0.2 mL of distilled water was added. For 30 to 60 seconds, the tube was vortexed to create a consistent suspension. Each slope of the test media containing medication in varying concentrations was infected with one loopful (10 µL) of this suspension. After four weeks of incubation, these injected slopes were monitored for growth at 37 °C. In the absolute concentration method, the slopes containing at least or more than 20 colonies were defined as growth.

Interpretation

Each slope was examined carefully and the results were recorded as follows;

- **3+:** Growth which is confluent (like a lawn culture)
- **2+:** Innumerable colonies (if there were more than 100 colonies on the slope)
- **1+:** 20–100 colonies (counted approximately)
- **1–19:** Exact number of colonies

The Minimum Inhibitory Concentration (MIC) was the lowest concentration of the drug-containing medium that prevented the organism from growing. The minimum concentration of growth inhibition at which the isolates were considered to be resistant (MIC) was set as 32 g/mL for RMP and 0.2 g/mL for INH.

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MTT Method[16,17]

Prior to testing the isolates, the inoculum size was standardised, and the incubation time needed for both INH and RIF sensitivity tests was optimised. The test medium, Middle Brook 7H9 broth, was enhanced with 0.01% glycerol (HiMedia - FD329-5VL), 10% OADC (oleic acid, albumin, dextrose, and catalase) and (3-4, 5-dimethylthaizol-2-yl-2, 5-diphenyl tetrazolium bromide) (SIGMA-M2128) MTT solution.

Inoculum Size Standardisation

A suspension of 8 mL was prepared as inoculum in 7H9 medium from LJ slopes which were three to four weeks old each for sensitive control strain (SC), resistant control strain (RC) and standard sensitive H37Rv strain (SS). The standard strains were obtained from the National Institute for Research in Tuberculosis (NIRT), Chennai. A serial tenfold dilution was made for different concentrations ranging from 10^7 cfu/mL to 10^5 cfu/mL for each strain. After dispensing 1 mL of each strain's 8 mL suspension into seven screw-cap sterile tubes, the strains were cultured for seven days at 37 °C. The MTT assay method was used to examine one tube from each concentration after 24 hours and then daily after that. They were inspected for the development of purple colour. The concentration of 10^7 cfu/mL was determined to be the standard inoculum size for evaluating additional samples because this experiment demonstrated that it would produce a colour change as soon as possible.

Inoculum Time Standardisation

Before beginning the MTT experiment, the incubation times for each drug were optimised by arranging four sets of four tubes and incubating them at 37 °C for two to six days for RIF and four to nine days for INH. The ultimate concentration of RMP was 1 µg/mL after 0.5 mL of standardised inoculum and 0.5 mL of RIF solution, which contained a concentration of 2 µg/mL, were combined. For INH, 0.1 mL of standardised inoculum was added to a 0.9 mL solution (0.4 mL plain 7H9 and 0.5 mL drug medium) in order to reach a final drug concentration of 0.2 µg/mL. Two tubes, one for each of the tested resistant and sensitive isolates, contained 0.5 mL of the standard inoculum and 0.5 mL of plain 7H9 as drug-free controls. In order to test for colorimetric readings, control tubes were set up as blank controls with just media and drug solution.

MTT Assay

To carry out the assay Each tube received 10 µL of MTT solution, which was then incubated at 37 °C for four hours. The tubes were then filled with 1 mL of a solubilising solution that contained 0.1 N HCL in isopropanol, and the tubes were inverted to ensure that the contents were well combined. If the strains in the control and drug-containing tubes changed colour to purple after 30 to 60 minutes of room temperature incubation, it was identified as resistant. The strain was identified as sensitive when it changed colour in drug-free controls but remained unchanged in the drug-containing tube.

Results

Drug susceptibility tests for two medications, INH and RMP, were performed on the 71 isolates using both the absolute concentration method and the MTT method. 94% of isolates, according to the results, were susceptible to both medications. Of the eleven isolates that were discovered to be resistant, five were found to be resistant to both RMP and INH. More than 20 colonies grew (Figure 1), with MICs of ≥ 0.2 µg/mL for INH and ≥ 32 µg/mL for RMP, these isolates were classified as MDR strains.

Absolute Concentration Method

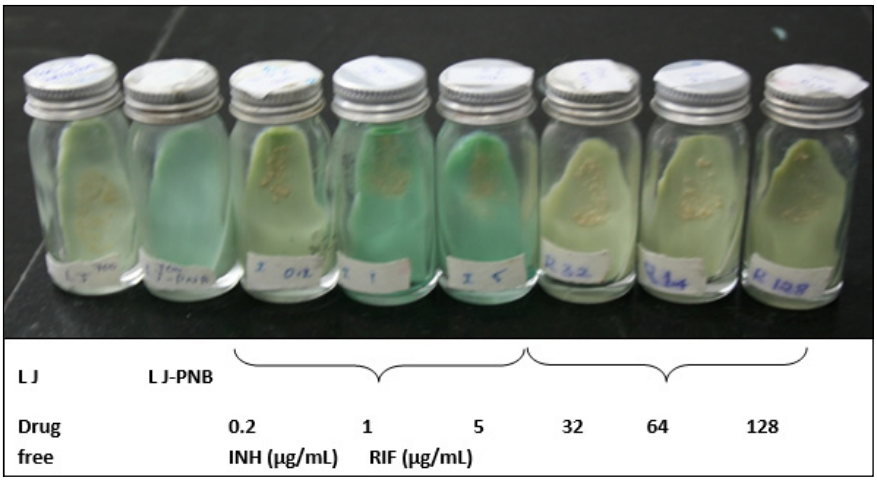

Table 1.Literature Review for Shigellosis

L J: Lowenstein Jensen medium

L J – PNB: Lowenstein Jensen medium with para nitro benzoic acid

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Table 1.Resistance Pattern by Absolute Concentration Method and MTT Assay

Evaluation of Drug Sensitivity Tests	Rifampicin				Isoniazid			
	Absolute concentration method 32 µg/mL		Assay by MTT 2 µg/mL		Absolute concentration method 0.2 µg/mL		Assay by MTT 0.4 µg/mL	
	R	S	R	S	R	S	R	S
	06	65	07	64	10	61	09	62
Total	71		71		71		71	

The results of the resistance pattern obtained by MTT assay and absolute concentration methods were similar. Using both techniques, five MDR isolates were found; nevertheless, the mono resistance pattern in both medications differed negligibly. The number of isolates detected to be mono-resistant to INH and RMP were 4 and 2 respectively by MTT assay compared to 5 and 1 by absolute concentration method (Table 1).

Repeated Measures ANOVA

There was no significant difference between within-group effects with a p value of 0.12 (> 0.05). No significant difference was found between the groups, with a p value of 0.09 (> 0.05). The sphericity assumption was not satisfied hence Greenhouse-Geisser effect was considered. The test compared the absolute concentration method and MTT assay within each group and between RMP and INH.

Discussion

There are a few studies in which different methods of antimicrobial susceptibility of M. tuberculosis have been compared. We tested 71 isolates of *Mycobacterium tuberculosis* and found good agreement between the two methods used in the present study. The percentage technique is the gold standard for drug susceptibility testing, according to the National Committee for Clinical Laboratory Standards (NCCLS) standards[18] (formerly the Clinical and Laboratory Standards Institute, or CLSI). Because it was technically straightforward to prepare the inoculum and analyse the data, the absolute concentration approach was chosen.[19] Heifets et al. state that if it is feasible to standardise the crucial drug concentrations in a lab, the absolute concentration technique can be applied.[20] In our laboratory, the absolute concentration method was standardised with a sensitive strain (H37Rv) and a standard MDR strain. The MTT assay is a rapid colorimetric assay. In our study, good sensitivity was shown by this assay for both INH and RMP. Their accordance points to detect resistance were adequately high to both drugs - RMP and INH. As compared to other studies, our study showed a sensitivity of 100% to RMP and 96% to INH.[17,18,21,22] In a different investigation, the MTT assay was also employed directly on sputum samples to detect RMP resistance. The results were achieved quickly—within two weeks—and matched the absolute concentration technique by 98.5%.[23]

The data analysis from different studies in our country showed that the primary resistance to first-line drugs ranged from 0.0% to 3.4%. This was equivalent to the data (1%) of our study. The data from our study on primary MDR-TB was also found to be reliable with a median prevalence of 1.1% (range 0.0–14.2%) worldwide.[24] The aggregated data of M. tuberculosis drug resistance received by WHO globally from 156 countries during the period between 2003 and 2017 showed an overall prevalence of INH resistance (with or without concomitant RMP resistance) range between 10.7% (9.6–11.9) and 27.2% (24.6–29.9).[25] The response in our study was also similar with a comparable response of higher resistance to INH (10%) than RMP which showed 6% resistance. This data signifies the need to test both the first-line drugs to determine drug resistance as both – INH and RMP are powerful first-line medicines and resistance to either will be detrimental to the treatment outcome as a failure, relapse or development of resistance to other anti-tuberculous drugs.

Mycobacterium tuberculosis is a slow-growing organism, laboratory identification by absolute concentration method can take as long as eight to ten weeks but the MTT assay gives results in two weeks.

Conclusion

With the increasing number of resistant and MDR-TB strains, there is an essential demand for rapid, appropriate and accurate drug susceptibility testing. In an upcoming era of rapid diagnosis by molecular methods, the MTT assay was tested to see if it could replace the absolute concentration method as a preliminary drug susceptibility testing method in remote areas without access to diagnostic methods

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like GeneXpert. Though there is a rapidity of results, the accuracy of detecting drug resistance is not precise enough to benefit the patients. Hence we concluded that the absolute concentration method is better than the MTT assay in terms of simplicity of performance and accuracy of results though the MTT assay may be used as an interim test for drug susceptibility.

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Authors' Contribution:

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process: None

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