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Determination of ethambutol MICs for *Mycobacterium tuberculosis* and *Mycobacterium avium* isolates by resazurin microtitre assay

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***Objectives*: To test susceptibilities of *Mycobacterium tuberculosis* (MTB) isolates to ethambutol by the Löwenstein–Jensen (LJ) proportion method and resazurin microtitre assay (REMA) and to evaluate REMA for the determination of ethambutol MICs for MTB and *Mycobacterium avium* isolates.**

***Methods*: A total of 50 MTB and 20 *M. avium* isolates were tested to determine the MICs of ethambutol by REMA and agar dilution method. MTB isolates were also tested by the LJ proportion method.**

***Results*: REMA provided ethambutol susceptibility results for all the isolates within 8–9 days. For MTB isolates, REMA showed 96.7% sensitivity, 100.0% specificity and 98.0% accuracy when LJ proportion results were taken as 'gold standard'. For both MTB and *M. avium* isolates, the MICs determined by REMA were lower than those determined in agar medium, indicating that MIC values determined by REMA are closer to the actual MICs for the isolates.**

***Conclusions*: REMA can be used as a rapid and inexpensive method for mycobacterial drug susceptibility testing against ethambutol. In comparison with the agar method, the MICs determined by REMA can more accurately be correlated with achievable plasma concentrations of antimycobacterial agents.**

Keywords: mycobacteria, drug susceptibility, *M. tuberculosis*, *M. avium*

Introduction

Ethambutol is a narrow-spectrum antimycobacterial agent that is used for the treatment of tuberculosis as well as infections caused by *Mycobacterium avium*. Ethambutol has been shown to have bactericidal action against *Mycobacterium tuberculosis* (MTB) and *M. avium*.[1] Ethambutol is an important antimycobacterial drug as it enhances the effect of other companion drugs including aminoglycosides, rifamycins and quinolones. Moreover, this drug has also been shown to significantly decrease the levels of bacteraemia in patients with AIDS,[2] showing its potential role in treatment of infections caused by *M. avium* strains.

Availability of rapid results of mycobacterial culture and drug susceptibility testing is a prerequisite for the design and success of treatment regimens. Conventional methods available for mycobacterial drug susceptibility testing are based on solid media that take longer turnaround times to give final results. Various rapid methods have also been developed for this purpose, but these methods require expensive instruments and/or tedious procedures. Palomino *et al.*[3] had proposed an assay based on the oxidation–reduction dye resazurin, called the resazurin microtitre assay (REMA), to test drug susceptibilities of MTB isolates. This assay is simple, inexpensive and gives rapid results in comparison with conventional methods. REMA has been used in various studies to test susceptibilities of MTB isolates to rifampicin and isoniazid. However, the experience about its performance with other first-line antitubercular drugs, such as ethambutol, is limited.[4] We have used REMA for the determination of MICs of ethambutol for MTB and *M. avium* isolates. Objectives of the present study were: (i) to compare ethambutol susceptibility of MTB isolates by the Löwenstein–Jensen (LJ) proportion method and REMA; and (ii) to evaluate the utility of

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MIC determination of ethambutol for MTB and *M. avium* isolates by REMA and the agar dilution method.

Materials and methods

Mycobacterial isolates

A total of 50 MTB and 20 *M. avium* isolates were analysed for their susceptibilities to ethambutol. All the mycobacterial isolates included in this study were from the collection of the Mycobacterial Repository Centre of our institute. MTB isolates were obtained from sputum samples of pulmonary tuberculosis patients collected during the period September 2004–August 2005 from different parts of India. *M. avium* isolates were from clinical (sputum) as well as environmental (soil and water) sources collected from the Agra region. Isolates were maintained on LJ medium and freshly subcultured before being used for further microbiological investigations.

Susceptibility testing by the proportion method

MTB isolates were tested for their susceptibilities to ethambutol (Sigma Chem. Co., USA) by the standard proportion method on LJ medium.[5] Briefly, a bacterial suspension was prepared and several 10-fold dilutions (10^{-1}, 10^{-2} and 10^{-3}) of this suspension were inoculated on drug-containing (critical ethambutol concentration was 2 mg/L) and drug-free LJ slopes. Media bottles were incubated at 37°C. Final readings were noted after 6 weeks and an isolate showing 1% or more growth on ethambutol-containing slopes in comparison with drug-free slopes was considered resistant, otherwise susceptible.[6]

MIC determination by the REMA plate method

The REMA plate method was performed as described previously[3] to determine the MICs of ethambutol for all the mycobacterial isolates. Briefly, a 100 μL volume of Middlebrook 7H9 broth (Difco, USA) was dispensed in each well of a 96-well cell culture plate (Nunc, Denmark). Ethambutol concentrations prepared directly in the medium were 1.25, 2.5, 3.75, 5.0, 6.25, 7.5, 8.75 and 10.0 mg/L. Perimeter wells of the plate were filled with sterile water to avoid dehydration of the medium during incubation. A standard bacterial suspension equivalent in turbidity to that of a no. 1 McFarland standard was prepared and diluted 1:20 in 7H9 broth; a 100 μL inoculum was used to inoculate each well of the plate. A growth control containing no ethambutol and a sterile control without inoculum were also included for each isolate. Plates were sealed and incubated at 37°C for 1 week. Twenty-five microlitres of 0.02% resazurin (Sigma Chem. Co.) solution was added to each well; plates were re-incubated for an additional 2 days. A change in colour from blue to pink indicated the growth of bacteria, and the MIC was read as the minimum ethambutol concentration that prevented the colour change in resazurin solution. It is important to note that we have used the final ethambutol concentration of each well (by taking half values of the ethambutol concentrations prepared initially in the wells of the microtitre plates) during interpretation of the results.

MIC determination by the agar dilution method

MTB and *M. avium* isolates were tested on Middlebrook 7H10 and 7H11 agar media (Difco), respectively, to determine their MICs of ethambutol as per the described procedure.[1] Ethambutol concentrations prepared in agar media were 0, 1.0, 5.0, 7.5, 10.0, 15.0, 20.0 and 30.0 mg/L. A standard bacterial suspension equivalent in turbidity to that of a no. 1 McFarland standard was prepared and diluted 1:100. One hundred microlitres of this diluted suspension was used to inoculate agar media. Final readings were taken after 3 weeks of incubation at 37°C. MIC was defined as the minimum ethambutol concentration resulting in no visible growth on agar medium.

Costs

Costs (shipping, custom taxes and labour not included) involved in the tests were calculated by reviewing purchase records or catalogue prices from commercial suppliers.

Results and discussion

Among the 50 MTB isolates, 19 were ethambutol-susceptible and 31 were ethambutol-resistant by the LJ proportion method. The MICs for MTB and *M. avium* isolates determined by REMA are presented in Table 1. Ethambutol-resistant and ethambutol-susceptible MTB isolates showed a clear distinction between their MICs in REMA. Ethambutol MICs for MTB isolates determined to be resistant by the proportion method were at least 3.125 mg/L (except for one isolate with an MIC value of 2.5 mg/L). Ethambutol MICs of 2.5 mg/L or lower were found in REMA for isolates determined to be susceptible by the proportion method. On the basis of these results, the tentative breakpoint concentration for ethambutol resistance was defined as 3.125 mg/L in REMA. At the proposed breakpoint

Table 1. Results of ethambutol susceptibility testing for MTB and *M. avium* isolates by REMA

	No. of isolates	Cumulative percentage of isolates inhibited at MIC (mg/L) of								
		0.625	1.25	1.875	2.5	3.125	3.75	4.375	5.0	>5.0
MTB										
EMB-susceptible[a]	19	31.6	57.9	78.9	100	—	—	—	—	—
EMB-resistant[a]	31	0	0	0	3.2	16.2	19.4	29	38.7	100
M. avium	20	0	15	20	20	20	25	35	35	100

EMB, ethambutol.
[a]Results are based on the LJ proportion method.

concentration, out of 31 ethambutol-resistant isolates, only one MTB isolate (3.2%) was misclassified as susceptible in REMA. The proportion of resistant population on LJ medium showed that the isolate showing the discordant result was a case of borderline resistance as only 1.04% population of this isolate was resistant to ethambutol. Taking LJ results as the 'gold standard', accuracy, sensitivity and specificity of REMA were calculated to be 98.0%, 96.7% and 100.0%, respectively. Similarly, predictive values of susceptibility and resistance for REMA were 95.0% and 100.0%, respectively.

Previously, Montoro *et al.*[4] had suggested a 4 mg/L ethambutol concentration (initially prepared in the wells of microtitre plates) as the cut-off in REMA; however, at this cut-off, the specificity of the assay was very low. In our study, at a 3.125 mg/L cut-off for ethambutol (equivalent to 6.25 mg/L ethambutol prepared initially in the microtitre well), we obtained 100% specificity without compromising sensitivity. Early determination of ethambutol MICs for MTB isolates is valuable as a large proportion of high-level ethambutol-resistant MTB isolates have also been reported to be isoniazid resistant.[7] This observation needs to be studied in different settings. Our results also implicate that, by testing narrow differences in drug concentrations, exact cut-off levels for antitubercular drugs can be determined. This approach can be applied in future studies for testing susceptibilities to other antitubercular drugs, such as streptomycin, whose results have been shown to display low reliability.[4]

In this study, REMA results were available for all the isolates after 2 days of addition of resazurin solution. The current estimated cost for ethambutol susceptibility testing per sample with REMA is $0.576, which is about 10 times higher than the LJ proportion method (Table 2). However, REMA can be performed with minimum labour inputs when compared with the other two methods used in this study. The cost of REMA can be reduced further by decreasing the number of tested drug concentrations, such as one optimum concentration (i.e. 5 mg/L ethambutol) used previously by Palomino and Portaels[8] in a simplified microplate Alamar Blue assay (MABA). One disadvantage of REMA is associated with biosafety since the plates use liquid medium and could generate aerosols. However, these concerns can be taken care off by adapting this assay to screw-cap tube format.

The MICs determined for *M. avium* isolates by REMA were 1.25 mg/L for 15.0%, 1.875 mg/L for 5.0%, 3.75 mg/L for 5.0%, 4.375 mg/L for 10.0% and >5 mg/L for 65.0%. When these MICs were compared with the 3.125 mg/L breakpoint concentration adopted for MTB, most of the *M. avium* isolates fell into the resistant group (Table 1). However, it has been previously reported that in contrast to MTB isolates, *M. avium* isolates show broad ranges of MICs of various drugs.[9] Hence, *M. avium* susceptibility testing based on the breakpoint concentration adopted for MTB will have little clinical significance. However, correlation of the ethambutol MICs with peak plasma levels of ethambutol (2–5 mg/L)[6] showed that 20% of isolates (ethambutol MICs up to 1.875 mg/L) could be grouped as susceptible, 15% of isolates (MICs within the range of 3.75–4.375 mg/L) as moderately susceptible and 65% of isolates (MICs more than 5 mg/L) as resistant. Hence, interpretation of ethambutol MICs in comparison with the achievable plasma concentration and therapeutic response would be more useful for assessing the susceptibilities of *M. avium* isolates.

Table 2. Comparison of cost involved in ethambutol susceptibility testing by different methods

Susceptibility method	Estimated cost ($)/ isolate	Labour involved
LJ proportion	0.058	high
REMA	0.576	low
Agar dilution	4.451	medium

Previously, Vanitha and Paramasivan[10] reported usefulness of MABA for drug susceptibility testing of *M. avium* isolates against clarithromycin. Our study showed that REMA can also be used for drug susceptibility testing of *M. avium* isolates. In contrast to Alamar Blue, resazurin is a non-proprietary compound and hence REMA would be an inexpensive alternative to MABA for susceptibility testing of *M. avium* isolates.

In our study, for those mycobacterial isolates having their ethambutol MICs within the tested concentration range, the broth-determined MICs were usually lower than the agar-determined MICs (data not shown). The only exception was one MTB isolate with an MIC of ethambutol of 5 mg/L by both methods. These data are in agreement with a previous investigation by Heifets *et al.*[1] on ethambutol. The differences in the MICs are attributed to higher levels of absorption, binding and degradation of the drug in solid media, particularly because of the longer incubation period required for obtaining sufficient growth in solid media.[6] Moreover, drug activity is more pronounced in liquid medium because drug remains in direct contact with bacterial growth. It is because of these reasons that broth-determined MICs of antimycobacterial agents are more accurate and closer to the true MICs and have been suggested to be more accurately correlated with their achievable plasma concentrations.[6]

Tentative criteria for mycobacterial susceptibilities have been proposed on the basis of the comparison of the MICs determined radiometrically in 7H12 broth and achievable drug levels in serum.[6,9] Our results showed that the REMA plate method can also be used to establish such tentative criteria. More importantly, we suggest careful correlation between the MICs determined by REMA and the achievable plasma concentrations as the actual MIC values must be read for such comparisons. Quantitative measurement of degree of susceptibility can help in management of infection as an increase in MIC during the course of chemotherapy may be an indirect marker that a drug is indeed affecting the bacterial population.[9] However, the exact clinical relevance of MIC determination for monitoring the treatment of mycobacterial diseases can only be established on the basis of clinical trials.

In summary, REMA was able to overcome the problems of longer turnaround times and higher MICs associated with drug susceptibility testing on solid media by giving rapid results and utilizing Middlebrook 7H9 broth as culture medium. REMA could be particularly useful for proper management of mycobacterial infections in low resource countries as it provides more accurate MICs at a reasonable cost.

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Transparency declarations

None to declare.

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