

Microplate nitrate reductase assay versus Alamar Blue assay for MIC determination of *Mycobacterium tuberculosis*

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SUMMARY

A rapid colorimetric, microplate based nitrate reductase assay (NRA) method for minimum inhibitory concentration (MIC) determination of clinical isolates of *Mycobacterium tuberculosis* was compared with the microplate Alamar Blue assay (MABA), presently in vogue.

Reproducible results were obtained in 7 days by NRA. The NRA method was found to be inexpensive, suitable for MIC determination against *M. tuberculosis* and can be suggested as an ideal substitute to MABA.

KEY WORDS: MIC; NRA; *Mycobacterium tuberculosis*

TUBERCULOSIS (TB) remains the largest infectious cause of human mortality even decades after the introduction of effective chemotherapy.¹ A number of factors are thought to account for the resurgence of TB. These include the acquired immune-deficiency syndrome (AIDS) epidemic, immigration from areas of high endemicity, transmission in high-risk environments, and increase in the number of multidrug-resistant (MDR, defined as resistance to at least isoniazid [INH] and rifampicin [RMP]) strains of *Mycobacterium tuberculosis*.

Standard methods for the diagnosis and drug susceptibility testing (DST) of *M. tuberculosis*, such as the proportion method and the absolute concentration method, are used globally, but they depend on growth of culture on solid media and are therefore time consuming.^{2,3} Other methods, such as the BACTEC 460 TB system,⁴ and oxidation-reduction dyes, e.g., tetrazolium⁵ and microplate Alamar Blue assay (MABA),^{6,7} are faster but have the drawback of requiring either radioactivity or expensive substrates, and are consequently not feasible in most resource-poor settings. A cost-effective and rapid drug susceptibility method is required to guide TB treatment.

M. tuberculosis possesses the ability to reduce nitrate to nitrite; this property is routinely used for biochemical identification of mycobacterial species. In the nitrate reductase assay (NRA), the presence of nitrite can easily be detected with specific reagents that produce a colour change.⁸ In the present study, we have employed NRA for rapid detection of minimum inhibitory concentration (MIC) in broth dilution assay.

Fifty-two clinical isolates of *M. tuberculosis* on

which DST was performed were obtained from the national repository of *M. tuberculosis*, Jalma Hospital, Agra, India. This also included *M. tuberculosis* H₃₇R_V (ATCC 27294), used as the quality control strain in this study. Middlebrook 7H9 medium with 1000 µg/ml sodium nitrate (NaNO₃) was used for NRA; 100 µl of sterilised Middlebrook 7H9 broth supplemented with 10% albumin-dextrose-catalase (ADC) and 0.05% Tween 80 was dispensed in each well of a sterile 96-well plate (U-bottom, Tarsons, Kolkata, India), and serial two-fold dilutions of drugs were prepared directly on the plate by adding 100 µl of the working solution of drug to achieve the final concentration. The following concentrations of drugs were used: 0.03 to 16 µg/ml (0.03, 0.06, 0.12, 0.25, 0.5, 1, 2, 4, 8, 16 µg/ml) for INH and RMP; and 0.12 to 64 µg/ml (0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 µg/ml) for ethambutol (EMB).

The inoculum was prepared from the 10-day-old cultures grown in Middlebrook 7H9 broth, supplemented with 10% ADC and 0.05% Tween 80. The turbidity of the cultures was adjusted to McFarland standard no. 1, further diluted to 1:50, and 100 µl of this inoculum was added to the wells of the 96-well plate. A growth control well and a sterile control were also included for each isolate. All plates were sealed with cling film and were incubated at 37°C in 5% CO₂ for 7 days. The NRA reagent was prepared by mixing one part of 50% (vol/vol) hydrochloric acid (Rankem, New Delhi, India), two parts of 0.2% (wt/vol) sulphanic acid (Hi-media, Mumbai, India), and two parts of 0.1% (wt/vol) 1-naphthylamine (Hi-media) just before use. After an incubation period of

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Table 1 MIC result of *M. tuberculosis* isolates for INH, RMP and EMB as determined by NRA

Drugs	Susceptible strains				Resistant stains			
	Cultures <i>n</i>	MIC ₅₀ µg/ml	MIC ₉₀ µg/ml	MIC range µg/ml	Cultures <i>n</i>	MIC ₅₀ µg/ml	MIC ₉₀ µg/ml	MIC range µg/ml
INH	39	0.06	0.25	0.06–0.25	14	8	>16	8–>16
RMP	43	0.12	0.25	0.06–0.50	10	>16	>16	8–>16
EMB	47	2	4	1–4	6	32	>64	32–>64

MIC = minimum inhibitory concentration; INH = isoniazid; RMP = rifampicin; EMB = ethambutol; NRA = nitrate reductase assay.

Table 2 MIC result of *M. tuberculosis* isolates for INH, RMP and EMB as determined by MABA

Drugs	Susceptible strains				Resistant stains			
	Cultures <i>n</i>	MIC ₅₀ µg/ml	MIC ₉₀ µg/ml	MIC range µg/ml	Cultures <i>n</i>	MIC ₅₀ µg/ml	MIC ₉₀ µg/ml	MIC range µg/ml
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MIC = minimum inhibitory concentration; INH = isoniazid; RMP = rifampicin; EMB = ethambutol; MABA = microplate Alamar Blue assay.

7 days, 35 µl NRA reagent was added to one of the positive control wells in column 11. If the contents of the well turned pink, the reagent was added to all wells of the 96-well plate. The results were recorded as: negative (no colour change) or positive (pink to deep red).

Drug susceptibility was also determined by MABA.⁶ Briefly, drug dilutions and inoculum preparation were performed as described above in Middlebrook 7H9 broth, supplemented with 10% ADC. On day 7 of the incubation, 50 µl of freshly prepared 1:1 mixture of 10 × Alamar Blue reagent (Accumed International, Westlake, OH, USA), and 10% Tween 80 was added to one well among the positive controls. The plates were further incubated at 37°C for 24 h. If the contents of the well turned pink, the reagent mixture was added to all the wells of the microplate.

The results were expressed in terms of MIC₅₀ (at which 50% of the isolates were inhibited) and MIC₉₀ (at which 90% of the isolates were inhibited). This study demonstrated excellent agreement between the results obtained by NRA and MABA methods, with results available in 8 days for all the 52 isolates tested, as summarised in Table 1 and Table 2, respectively. There was complete agreement between the results obtained by NRA and MABA for RMP and EMB. However, agreement between NRA and MABA was 96% for INH. The MIC of two susceptible strains was 0.06 µg/ml and 0.125 µg/ml, respectively, by NRA, whereas these two strains showed an MIC of 0.03 µg/ml by MABA (data not shown).

The ability to reduce nitrate is typical for *M. tuberculosis*, and nitrate reductase-negative strains of *M. tuberculosis* are very unusual.⁹ Lemus et al. used NRA for DST by incorporating KNO₃ in Löwenstein-Jensen (LJ) medium along with the drug before inspersion.⁹ However, in the present study, we extended the application of NRA from LJ medium to Middle-

brook 7H9 liquid medium for the determination of colorimetric MIC of *M. tuberculosis* isolates.

The NRA described here is less cumbersome, as the detection of colour change is instant as compared to the overnight incubation required in MABA and other tetrazolium dyes. The results of the present study were not compared with the proportionate agar method or absolute concentration method, as MIC determination using MABA and other tetrazolium salts has already been reported and amply compared with other gold standards such as the proportion method.^{5,6} Moreover, the objective of this study was to suggest a specific and inexpensive alternative to MABA and other tetrazolium dyes.

In comparison to the other methods, NRA is more cost-effective for developing countries. It also provides a wide range of precise MICs, which can be used to define susceptibility breakpoints for the older drugs in current use as well as to establish breakpoints for newer agents. There are biosafety concerns associated with both of these methods, as using liquid medium in the microtitre plates with *M. tuberculosis* is prone to create aerosols. However, this format can be adapted to screw-cap containers to avoid this situation.⁹

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