INT J TUBERC LUNG DIS 9(8):939–941

SHORT COMMUNICATION

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

M. Kumar, I. A. Khan, V. Verma, G. N. Qazi

Biotechnology Division, Regional Research Laboratory, Jammu Tawi, India

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., tetrazolium[5] 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* $H_{37}R_V$ (ATCC 27294), used as the quality control strain in this study. Middlebrook 7H9 medium with 1000 μg/ml sodium nitrate ($NaNO_3$) 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% CO_2 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) sulphanilic 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

Correspondence to: Dr Inshad Ali Khan, Biotechnology Division, Regional Research Laboratory (CSIR), Canal Road, Jammu Tawi 180001, India. Tel: (+91) 191-2571163. Fax: (+91) 191-2543829, 2547850. e-mail: inshad@rrljammu.org or inshad@yahoo.com

Article submitted 17 September 2004. Final version accepted 24 December 2004.

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

	Susceptible strains				Resistant stains			
Drugs	Cultures *n*	MIC_{50} μg/ml	MIC_{90} μg/ml	MIC range μg/ml	Cultures *n*	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

	Susceptible strains				Resistant stains			
Drugs	Cultures *n*	MIC_{50} μg/ml	MIC_{90} μg/ml	MIC range μg/ml	Cultures *n*	MIC_{50} μg/ml	MIC_{90} μg/ml	MIC range μg/ml
INH	39	0.06	0.25	0.03–0.25	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; 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 MIC_{50} (at which 50% of the isolates were inhibited) and MIC_{90} (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 KNO_3 in Löwenstein-Jensen (LJ) medium along with the drug before inspissation.[9] However, in the present study, we extended the application of NRA from LJ medium to Middlebrook 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]

Acknowledgements

We thank Jalma Hospital, Agra, and Choitram Hospital, Indore, for providing us with the clinical isolates of *M. tuberculosis*.

This activity was supported by research grant from Council of Scientific & Industrial Research (CSIR), India.

References

1 Raviglione M C, Snider D E, Kochi A. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. JAMA 1995; 273: 220–226.

2 Canetti G, Froman S, Grosset J, et al. Mycobacteria. Laboratory methods for testing drug sensitivity and resistance. Bull World Health Organ 1963; 29: 565–578.

3 Inderlied C B, Salfinger M. Antimicrobial agents and susceptibility tests: mycobacteria. In: Murray P R, Barron E J, Pfaller M A, Tenover F C, Yelken R H, eds. Manual of clinical microbiology. 6th ed. Washington, DC: ASM Press, 1995.
4 Siddiqi S H, Libonati J P, Middlebrook G. 1981. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol 1981; 13: 908–912.
5 Caviedes L, Delgado J, Gilman R H. Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*. J Clin Microbiol 2002; 40: 1873–1874.
6 Franzblau S G, Witzig R S, McLaughlin J C, et al. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J Clin Microbiol 1998; 36: 362–366.
7 Gomez-Flores R, Gupta S, Tamez-Guerra R, Mehta R T. Determination of MICs for *Mycobacterium avium-M. intracellulare* complex in liquid medium by a colorimetric method. J Clin Microbiol 1995; 33: 1842–1846.
8 Golyshevskaia V I, Korneev A A, Chernousova L N, et al. New microbiological techniques in diagnosis of tuberculosis. Probl Tuberk 1996; 6: 22–25.
9 Lemus D, Martin A, Montoro E, Portaels F, Palomino J C. Rapid alternative methods for detection of rifampicin resistance in *Mycobacterium tuberculosis*. J Antimicrob Chemother 2004; 54: 130–133.

RÉSUMÉ

Nous avons comparé une méthode colorimétrique rapide reposant sur une méthode du test de nitrate réductase (NRA) sur microplaque pour la détermination de la concentration minimale inhibitrice (CMI) dans les isolats cliniques de *Mycobacterium tuberculosis* avec le test à l'Alamar Blue sur microplaque (MABA), actuellement en vogue. Des résultats reproductibles ont été obtenus après 7 jours par NRA. La méthode NRA s'est avérée peu coûteuse, adaptée à la détermination de la CMI pour *M. tuberculosis* et peut être suggérée comme un substitut idéal pour la méthode MABA.

RESUMEN

En el presente estudio se compararon dos sistemas para determinar la concentración mínima inhibitoria (CMI) contra *Mycobacterium tuberculosis* : el ensayo de reducción del nitrato (NRA), utilizando el método colorimétrico rápido en microplacas y el método del azul de Alamar en microplacas (MABA), de uso muy frecuente en la actualidad. Con el método de la NRA se obtuvieron resultados reproducibles en 7 días. Se encontró que este método no es costoso, es apropiado para medir la CMI contra *M. tuberculosis* y puede recomendarse como un sustituto ideal al método MABA.