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SHORT COMMUNICATION

Rapid and simple MTT method for rifampicin and isoniazid susceptibility testing of *Mycobacterium tuberculosis*

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SUMMARY

The MTT method for rifampicin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* was developed by using bacterial suspension prepared from colonies on solid media. The MTT tube assay in 1 ml Middlebrook 7H9 broth was completed within 4 days for rifampicin (RMP) and within 7 days for isoniazid (INH). When MTT assay results with 279 *M. tuberculosis* clinical isolates were compared with those of the conventional proportion method on Löwenstein-Jensen medium, high specificity and sensitivity values of 100% and 94.1%, respectively, for RMP susceptibility testing, and 99.5% and 89.2%, respectively, for INH susceptibility testing were obtained. The accuracy of the MTT method for RMP and INH was >0.97 concordance with the proportion method. The MTT method is simple, inexpensive and rapid. The high level of agreement with the conventional proportion method suggests a potential to rapidly detect drug-resistant *M. tuberculosis* in developing countries, as only basic microbiological equipment is need.

KEY WORDS: *M. tuberculosis;* susceptibility testing; MTT

TUBERCULOSIS (TB) is a major public health problem in the world.[1–3] Rifampicin (RMP) and isoniazid (INH) are the most rapidly bactericidal of the first-line anti-tuberculosis drugs, and *Mycobacterium tuberculosis* isolates that are resistant to at least both of these antibiotics are classified as multiple drug-resistant (MDR). The spread of drug-resistant TB in the AIDS era poses a major public health crisis. Prevention of the occurrence and spread of MDR-TB is therefore a major priority of all TB control programmes.

The rapid and accurate susceptibility testing of *M. tuberculosis* is essential for effective patient treatment and to prevent transmission of the disease.[4] The two most widely accepted drug susceptibility tests for *M. tuberculosis* are the proportion method and the BACTEC 460; both compare bacterial growth in drug-containing medium with that in drug-free medium. In the proportion method, drug-resistant colonies are counted after 3–4 weeks of incubation,[1,5,6] whereas in the BACTEC method the rate and amount of $^{14}CO_2$ produced from metabolising bacteria are quantified by the BACTEC 460 instrument. The BACTEC 460 has been used as a rapid method to obtain results within 5–10 days in developing countries, but it requires the use of radio-isotope and a proprietary instrument, and thus is costly to perform.[7] For developing countries, it would be helpful to have a simple and inexpensive test that can rapidly detect MDR *M. tuberculosis* strains.

Colorimetric methods using Alamar blue or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) have been developed as alternative methods of rapidly and indirectly determining cellular growth based on metabolic activity.[8–11] The yellow MTT dye is reduced by dehydrogenase in living cells to produce purple MTT formazan, which can be solubilised and read visually or quantified by spectrophotometric measurement at 570 nm.[12,13]

As described by Mashana et al. and Abate et al., these dyes have been used to develop simple, rapid assays for rifampicin susceptibility testing of *M. tuberculosis*.[8,9] This paper describes the 1 ml MTT tube method for rifampicin (RMP) and isoniazid (INH) susceptibility testing and an evaluation of its efficiency with 279 clinical *M. tuberculosis* isolates. MTT was used to assess the viability of bacterial cells after exposure to RMP or INH. The results were compared with those obtained by the proportion method.

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MATERIALS & METHODS

Isolates and drug preparation

Two hundred and seventy-nine clinical isolates of *M. tuberculosis* (72 strains with known drug susceptibility patterns using the proportional method from the stock collection and 207 new clinical isolates) were obtained from the Mycobacteriology Laboratory of the Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok. *M. tuberculosis* H37Rv and K37 (resistant to RMP and INH, Korean Institute of Tuberculosis) were included as the reference sensitive and resistant strains, respectively. The isolates were subcultured on two Löwenstein-Jensen (L-J) medium (Becton Dickinson, Sparks, MD) bottles and incubated for 3 to 4 weeks at 37°C. Each culture was used in an independent blind manner by those performing the various assays (MTT and proportion) in order to avoid bias in the interpretation of results. Stock solutions of RMP and INH (Sigma Chemical Co., St. Louis, MO) were prepared at 20 mg/ml in dimethylsulphoxide (DMSO) and 10 mg/ml in distilled water, respectively, dispensed in 0.1 ml aliquots and stored at −70°C until use. Drug solutions were subsequently diluted in Middlebrook 7H9 broth (Difco, Detroit, MI) and supplemented with 10% (vol/vol) OADC (oleic acid, albumin, dextrose, catalase; Difco). This medium is referred to as M7H9 broth.

MTT method

The MTT susceptibility testing method was modified from the methods of Mshana et al.[8] and Abate et al.,[9] and performed in round, 13 × 100 mm tubes with screw-caps. Duplicate tubes were used in each test. Inocula were prepared by suspension of colonial growth from 3–4 week old L-J cultures in M7H9 broth to a turbidity equal to that of a no. 1 McFarland standard (10^7 *M. tuberculosis* colony forming units/ml).

For RMP-MTT susceptibility testing, 0.5 ml of inoculum was dispensed into tubes containing 0.5 ml of a 2 μg/ml RMP solution to achieve a final concentration of 1 μg/ml RMP in M7H9 broth. For INH-MTT susceptibility testing, 0.5 ml of 0.4 μg/ml INH was dispensed into each tube. To achieve a final INH concentration of 0.2 μg/ml, 0.4 ml of M7H9 broth was added, followed by the addition of 0.1 ml of inoculum. A drug-free control tube was included for each test as well as a tube containing media with drug but without inoculum. All tubes were incubated at 37°C in an ambient atmosphere.

MTT assay

The MTT assay was performed on day 4 for RMP and on day 7 for INH by adding 10 μl of MTT solution (5 mg/ml in phosphate buffered saline pH 7.2; Sigma) into each culture tube and incubating them for 4 h at 37°C. One millilitre of solubilising solution containing 0.1 N HCl in isopropanol was then added, and the contents were mixed thoroughly by inverting the tubes. After 0.5–1 h of incubation at room temperature, the (purple) colour in each tube was recorded and the optical density (OD) was measured with a spectrophotometer at 570 nm. Blanks for each test consisted of drug alone in M7H9 broth with MTT solution and solubilising buffer. Relative OD unit (RODU) was derived by dividing the OD of drug-containing tube by the OD of the control tube. From our preliminary experiment with known drug-resistant and sensitive strains, a value of <0.2 RODU was considered to be a sensitive result. This is also consistent with previous reports.[8,9] The presence of a purple colour in a drug-containing tubes was recorded as a resistant result by visual reading. Drug-sensitive *M. tuberculosis* H37RV and drug-resistant *M. tuberculosis* K37 strains were used as the experimental controls.

Optimisation of incubation time

The resistant reference *M. tuberculosis* K37 strain, the sensitive reference *M. tuberculosis* H37Rv strain, three drug-sensitive clinical strains (nos. 926, 872, 2602), and three drug-resistant clinical strains (nos. 13205, 8315, 10604) were used in this experiment. RMP and INH susceptibility testing were performed as described above. In order to optimise the incubation time, they were incubated at 37°C for 3, 4, 5 and 7 days for RMP testing and 3, 5, 7 and 9 days for INH testing prior to the MTT assay.

Percentage of detected drug-resistant cells in the MTT testing

To determine the ability of the MTT testing to detect resistant bacteria, increasing proportions (0 to 16%) of the resistant strain of *M. tuberculosis* K37 were added to the *M. tuberculosis* H37Rv sensitive strain cultures after incubation for 4 and 7 days for RMP and INH testing, as described in the susceptibility test, and were then processed for the MTT assays.

Proportion method

The conventional proportion method on L-J medium was performed as described.[5] Drug solutions were added to L-J medium prior to inspissation to achieve final concentrations of 40 μg/ml RPM and 0.2 μg/ml INH.

RESULTS

Preliminary experiments (data not shown) using strains of known INH and RMP susceptibility were conducted in order to standardise various parameters. A final MTT concentration of 0.05 mg/ml was found to be optimal for visual differentiation of the purple, reduced formazan product from the yellow

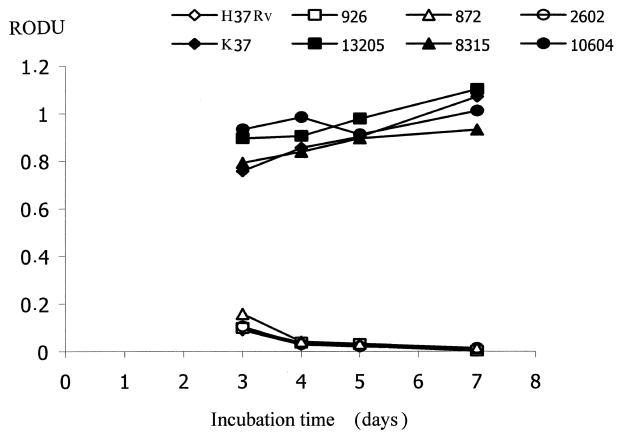

Figure 1 Influence of incubation time on RMP-MTT susceptibility testing of *M. tuberculosis* strains (H37Rv: sensitive reference; K37: resistant reference; 926, 872, 2602: sensitive clinical; and 13205, 8315, 10604: resistant clinical strains) in the presence of RMP (1 μg/ml). MTT reduction was determined following various days of incubation. RODU = relative optical density unit; RMP = rifampicin.

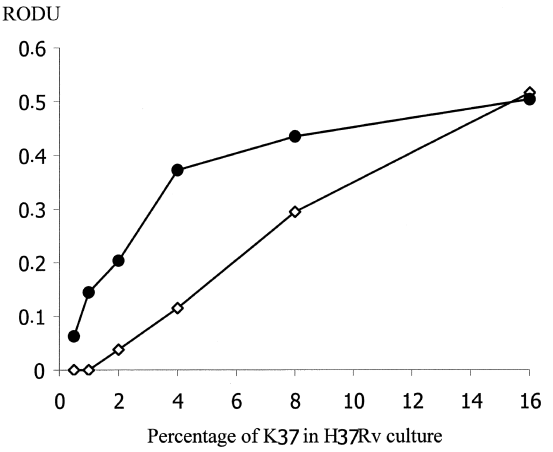

Figure 3 Threshold of detection of RMP and INH resistant subpopulation. Cultures containing various proportions of drug-resistant (K37) and drug-sensitive (H37Rv) *M. tuberculosis* reference strains. Bacteria were incubated in the presence of RMP (1 μg/ml) for 4 days (◇), or INH (0.2 μg/ml) for 7 days (●). RODU = relative optical density unit; RMP = rifampicin; INH = isoniazid.

parent MTT. For RMP susceptibility testing, the optimum inoculum was 0.5 ml of McFarland No. 1 cell suspension, in which case the assay could be completed by day 4 post-inoculation, with <0.2 RODU (Figure 1). For INH susceptibility testing it was necessary to use a lower inoculum size and to extend incubation to 7 days (Figure 2). By mixing various proportions of K37 reference drug-resistant and H37Rv reference drug-sensitive bacilli, it was determined that at least 2% and 8% of resistant strains could be detected at >0.2 RODU in the present of INH and RMP, respectively (Figure 3). These observations suggest that the drug-sensitive strains were detected by MTT method at <0.2 RODU at optimal conditions.

K37, H37Rv and the clinical isolates with known INH sensitivity and resistance were tested by the MTT method for INH susceptibility as described above, using INH concentrations of 0.2 and 1 μg/ml, which refer to the drug concentration of the standard proportion method on Middlebrook 7H10 agar medium.[14] Both the K37 and INH-resistant strains showed >0.6 RODU (range 0.68–0.97), and the H37Rv and INH-sensitive strains had <0.15 RODU (range 0.02–0.14) at both INH concentrations.

By visual reading of the MTT method, all drug-sensitive cultures had a light yellow appearance (OD < 0.08), and drug-resistant cultures varied from light to dark purple. Visual determinations of colour change (relative to that of purple in the control tubes) were correlated with RODUs. Cultures regarded as purple by visual observation had OD values of >0.1. RMP-resistant and INH-resistant isolates demonstrated RODU values of 0.82 ± 0.22, and 0.84 ± 0.25, respectively.

With regard to RMP susceptibility, there were a total of 276 (98.9%) concordant results between the MTT and the proportion methods (Table 1). Forty-eight of these isolates were resistant to RMP by both methods and 228 were susceptible. All three discordant isolates were resistant by the proportion method; two of these

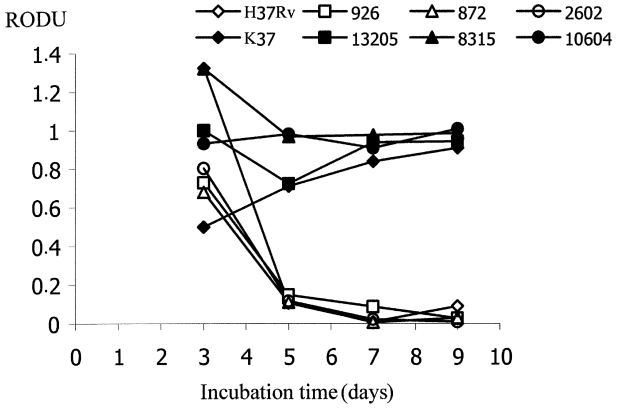

Figure 2 Influence of incubation time on INH-MTT susceptibility testing of *M. tuberculosis* strains (H37Rv: sensitive reference; K37: resistant reference; 926, 872, 2602: sensitive clinical; and 13205, 8315, 10604: resistant clinical strains), in the presence of INH (0.2 μg/ml). MTT reduction was determined following various days of incubation. RODU = relative optical density unit; INH = isoniazid.

Table 1 Comparison of RMP and INH susceptibility testing results. Number of indicated determinations by method

		Proportion method (μg/ml)			
		Rifampicin (40)		Isoniazid (0.2)	
Susceptibility		R	S	R	S
MTT method*	R	48	0	60	1
	S	3	228	5	213

* RMP concentration at 1 μg/ml, INH concentration at 0.2 μg/ml.
RMP = rifampicin; INH = isoniazid; S = susceptible; R = resistant.

Table 2 Efficiency of the MTT method in comparison with the proportion method

	Rifampicin	Isoniazid
Specificity (%)	100	99.53
Sensitivity (%)	94.12	92.30
Predictive value of susceptibility (%)	98.70	96.82
Predictive value of resistance (%)	100	97.71
Accuracy of MTT test*	0.99	0.98

* The probability of obtaining a true positive or true negative result.

showed resistant colonies on the drug-containing L-J media at 35% and 10% of the drug-free media.

When INH susceptibility testing results with the MTT method were compared with those obtained by the proportion method, a total of six (2.15%) discordant results were observed, five being resistant by the proportion method only and one by MTT only (Table 1). One of the discordant isolates showed 3% resistant colonies (data not shown) in the L-J proportion method but was sensitive by MTT methods. However, the MTT method did confirm the resistance of a strain with 10% resistant colonies by the proportion method (data not shown).

Specificity, sensitivity and predictive values for the MTT method were calculated as shown in Table 2. The MTT method had specificity and sensitivity values of 100% and 94.1%, respectively, for RMP susceptibility testing and 99.5% and 89.2%, respectively, for INH susceptibility testing. All predictive values were high for both the RMP and INH susceptibility tests (97–100%).

DISCUSSION

In previous studies,[8,9] it was not possible to use MTT to detect INH-resistant *M. tuberculosis*. In our preliminary experiments, INH susceptibility could not be evaluated after 4 days of culture but could be determined by extending incubation to 7 days. This prolonged incubation requirement for INH testing (relative to that required for RMP) was possibly due to the restricted activity of this drug against only actively replicating tubercle bacilli.[14]

Using the proportion method in M7H10 agar, the final drug concentrations of RMP and INH are 1 and 0.2 μg/ml, respectively. These drug concentrations were used in this MTT testing in M7H9 broth.

The sensitivity of the MTT method depends on the metabolic activity of viable cells in order to achieve measurable MTT reduction.[12,13] It has been suggested that drug-resistant isolates might have lower overall metabolic activity,[14] for example *rpoB* mutations leading to RMP resistance might effect the expression levels of particular enzymes. The experiments with mixtures of K37-resistant cells and H37Rv-sensitive cells suggested that an INH resistance level of 2% and an RMP resistance level of 8% or higher were required for detection by the MTT test. This lower metabolic activity among the resistant organisms could be seen in the results from isolates containing either 10% or 35% RMP-resistant cells detected by the L-J proportion method which were not detected by the MTT method in this experiment. In addition, the MTT method was capable of detecting one strain containing 10% INH-resistant bacteria but not at the level of 3% or more of the five resistant strains detected by the L-J proportion drug susceptibility method. The overall high concordance between MTT and the proportion method with respect to detection of rifampicin resistance among the clinical isolates suggests that most rifampicin-resistant cultures have a relatively high percentage of rifampicin-resistant bacilli.

MTT is readily soluble in water or phosphate buffer, and both powder and solutions are stable at 2–8°C for an extended period of time. It is less expensive than the Alamar blue reagent, and results can be determined visually as well as spectrophotometrically. As described here, the MTT drug susceptibility method can be performed easily in standard test tubes.

Although different quantities of inocula were required in this test, in actual practice this did not prove difficult as the aim was to obtain as early a result as possible for both RMP and INH. However, the best results were obtained for different incubation periods for the two agents. We are currently evaluating several experiments using an incubation period of 7 days for four anti-tuberculosis agents RMP, INH, streptomycin and ethambutol, in order to simplify and extend the range of sensitivities tested.

In conclusion, the MTT method is a simple, low cost susceptibility test for *M. tuberculosis* that can be completed in 4–7 days. Visual reading can be used to determine the growth of bacteria, but spectrophotometric reading provides more detailed information. Due to its high levels of agreement with the conventional proportion method, the MTT method has the potential to provide rapid detection of RMP and INH-resistant *M. tuberculosis* in clinical use. Optimisation of the MTT method for other antimicrobials may prove to be valuable for the routine susceptibility testing of *M. tuberculosis* against other anti-tuberculosis agents as well as for susceptibility testing of other mycobacterial species. Furthermore, the MTT methods should be adapted to use directly from acid-fast bacilli smear-positive specimens, to reduce the initial 3–4 week incubation period.

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RÉSUMÉ

La méthode MTT pour tester la sensibilité de *Mycobacterium tuberculosis* à la rifampicine et à l'isoniazide a été développée en utilisant des suspensions bactériennes préparées à partir de colonies provenant de milieux solides. Le test en tube MTT dans 1 ml de bouillon Middlebrook 7H9 a été terminé en 4 jours pour la rifampicine (RMP) et en 7 jours pour l'isoniazide (INH). Lorsque les résultats du test MTT sur 279 isolats cliniques de *M. tuberculosis* ont été comparés avec ceux obtenus par la méthode conventionnelle des proportions sur milieu de Löwenstein-Jensen, on a obtenu des valeurs élevées de spécificité et de sensibilité, respectivement de 100% et de 94,1% pour la RMP et respectivement de 99,5% et 89,2% pour l'isoniazide. La concordance de la méthode MTT avec la méthode des proportions pour la RMP et l'INH est supérieure à 0,97. La méthode MTT est simple, peu coûteuse et rapide. Le niveau élevé de concordance avec la méthode conventionnelle des proportions suggère des potentialités pour la détection rapide de *M. tuberculosis* résistant aux médicaments dans les pays en développement puisque l'équipement microbiologique de base suffit à la pratiquer.

RESUMEN

Se ha desarrollado el método MTT para los tests de sensibilidad de *Mycobacterium tuberculosis* a la rifampicina y a la isoniacida, utilizando suspensiones bacterianas preparadas a partir de colonias que crecen en medio sólido. El test en tubo MTT, en 1 ml de caldo Middlebrook 7H9, se realiza en 4 días para la rifampicina (RMP) y en 7 días para la isoniacida (INH). Cuando se compararon los resultados del test MTT en 279 aislados clínicos de *M. tuberculosis* con los resultados del método convencional de proporciones en medio Löwenstein-Jensen, se obtuvieron valores elevados de sensibilidad y de especificidad, de 100% y 94,1%, respectivamente, para los tests de sensibilidad a RMP y de 99,5% y 89,2%, respectivamente, para los tests de sensibilidad a INH. La concordancia del método MTT con el método de las proporciones para RMP e INH fue superior a 0,97. El método MTT es simple, barato y rápido. El alto nivel de concordancia con el método convencional de proporciones sugiere potencialidades para la detección rápida de *M. tuberculosis* resistente a los medicamentos en los países en desarrollo, dado que sólo se requiere el equipamiento microbiológico de base.