

Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*

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SUMMARY

OBJECTIVES: To standardise the colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for the rapid detection of rifampicin-resistant *Mycobacterium tuberculosis* in clinical practice and to evaluate the assay on a collection of 92 clinical isolates.

DESIGN: The Bactec method was used as the reference method. Rifampicin was used for the susceptibility testing in the Bactec method at a concentration of 2 µg/ml. The MTT assay was performed in tubes containing 3 ml Dubos broth; the assay is based on the principle that live cells convert the yellow tetrazolium salt into a blue formazan. A final concentration of 2 µg/ml rifampicin was used in the assay. Optical density (OD) values at 570 nm were recorded on the third and sixth day. A strain was defined as susceptible when the relative optical density

unit (RODU) (i.e., OD of rifampicin containing tube/OD of undiluted control) was ≤ 0.2 , and when the OD value of the rifampicin-containing tube on the sixth day was lower than the OD value on the third day. A strain was defined as resistant when the RODU was more than 0.5, and when there was an increase in OD value in the rifampicin-containing tube on the sixth day. The tubes were also read visually.

RESULTS AND CONCLUSION: The result obtained by the MTT assay perfectly matched the result obtained by the Bactec method. The MTT assay was also interpretable by the naked eye. This simple, inexpensive assay could be used as a rapid screening method for identification of rifampicin-resistant strains in low-income countries.

KEY WORDS: MTT; rifampicin resistance; *Mycobacterium tuberculosis*

MULTIDRUG-RESISTANT (MDR) tuberculosis (TB), defined as TB caused by *Mycobacterium tuberculosis* resistant to at least isoniazid (INH) and rifampicin, is an increasing health problem and a serious challenge to TB control programs.¹⁻³ Early diagnosis of MDR-TB is essential to prevent its transmission in the community. The traditional methods of culture and susceptibility testing using Löwenstein Jensen media or Middlebrook agar take 7 to 12 weeks.⁴ Most rapid methods for detection of MDR-TB, such as Bactec and molecular methods, are not affordable for routine use under program conditions in low-income countries.

The colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was introduced by Mossman⁵ as a quantitative measure of mammalian cell survival and proliferation, and was subsequently used to detect the viability of bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes* under adverse conditions.⁶ MTT is a yellow

tetrazolium salt that is converted into a blue formazan by dehydrogenases of a live cell. The assay is based on the principle that the amount of formazan produced is directly proportional to the number of live cells.^{5,6} The same principle also holds true for mycobacteria, and the assay has been described for susceptibility testing of *M. avium* complex (MAC) and *M. tuberculosis*.^{7,8} This study was conducted with the following objectives: 1) to standardise the MTT assay in a format appropriate for use in clinical practice in low-income countries, and 2) to evaluate the MTT assay for rapid detection of rifampicin resistance on a collection of *M. tuberculosis* strains.

MATERIALS AND METHODS

Strains

Ninety-two strains of *M. tuberculosis* were selected from the collection at Statens Serum Institute, Copen-

hagen. The strains originated from TB patients in Ethiopia, Denmark and Sweden. *M. tuberculosis* H₃₇Rv was included as the reference strain. Species identification was performed by DNA-RNA hybridisation (Accu Probe, GenProbe Inc, San Diego, CA) and by standard biochemical tests.⁹ Susceptibility testing was performed using the Bactec method (Becton Dickinson, Sparks, MD) with a final concentration of 2 µg/ml rifampicin.

Antibiotics and other chemicals

Rifampicin (Sigma Chemical Co., St. Louis, MO) was used to prepare a stock solution of 80 µg/ml in sterile water. The stock solution was aliquoted and kept at -20°C. A final concentration of 2 µg/ml rifampicin was used in all experiments.

A stock solution of MTT (Sigma) at a concentration of 6.7 mg/ml was prepared in phosphate buffer saline (PBS), pH 6.8, and was kept at 4°C in the dark. A final concentration of 0.5 µg/ml of MTT was used in the assay. A formazan solubilization buffer consisting of 17% sodium dodecyl sulfate (SDS, Merck-Schuchardt, Munich, Germany) in a 40% aqueous solution of dimethyl formamide (DMF, Sigma), was prepared by dissolving an accurately weighed SDS in the DMF solution at 37°C; the solution was kept at room temperature. The MTT solution was prepared every other week, and solubilization buffer was prepared every week.

Standardisation of inoculum size

A two-week-old culture of *M. tuberculosis* (strain R42/90) grown in Dubos broth was used to prepare four different bacterial suspensions with different turbidity. An aliquot of 500 µl from each suspension was added into three correspondingly labelled tubes (i.e., one each for the third, sixth and tenth day assay) containing 3 ml Dubos media, giving an approximate final bacterial concentration of 7×10^5 , 1.5×10^6 , 3×10^6 , 8×10^6 per ml.¹⁰ A 1:100 dilution of all the bacterial suspensions was also prepared in PBS; 500 µl of these suspensions were added to labelled tubes. Base line optical density (OD) at 570 nm (Novaspec II photometer, Pharmacia Biotech Ltd, UK) was measured on the same day. All tubes were incubated at 37°C until the MTT assay was performed on the third, sixth and tenth days.

The MTT assay was performed by adding MTT solution to the culture broth to give a final concentration of 0.5 µg/ml. The tubes were shaken and incubated at 37°C for 4 hours. A formazan solubilization buffer (500 µl) was added, and the tubes were mixed on vortex and incubated at 37°C for another hour. Absorbance was measured at 570 nm using a tube containing 3 ml Dubos, 500 µl PBS, MTT and solubilization buffer as a reference. All tubes were also compared with the reference tube to detect colour change by the naked eye.

The lowest bacterial concentration of a 1:100 diluted bacterial suspension which gave a measurable formazan formation on the third day, and its undiluted bacteria suspension, were chosen for further tests in the presence and absence of drugs.

Standardisation of the MTT assay for use in a tube format

The MTT assay was previously standardised for the detection of rifampicin resistance using a 96-well microtiter plate.⁸ Further standardisation was necessary to develop a more appropriate and safer protocol which uses test tubes. Two rifampicin-susceptible strains (R42/90 and R42/91) and two rifampicin-resistant strains (S:11/94 and S:293/91) grown in Dubos broth for two weeks were used for standardisation in a tube format. Two sets of tubes, one set for the third and another set for the sixth day assay, were used for each strain. Each set contained three tubes (i.e., one for undiluted control, one for 1:100 diluted control, and one for bacteria and rifampicin). All tubes contained 3 ml Dubos broth. An aliquot of 500 µl of bacterial suspension with turbidity corresponding to the turbidity of McFarland 2 (i.e., turbidity whose 1:100 dilution gave a measurable formazan formation on the third day) was added to the control tubes (control A) and to a tube containing rifampicin at a final concentration of 2 µg/ml. The same volume of 1:100 diluted bacterial suspension was added to correspondingly labelled control tubes (control B). All tubes were incubated at 37°C, the MTT assay was performed on the third and sixth days, as described above. Observation of the gross colour change in the rifampicin-containing tube as compared to the controls, and calculation of the relative optical density (RODU) (i.e., OD of rifampicin containing tube/OD of undiluted control) was made. A tube containing 3 ml Dubos broth, 500 µl PBS, MTT and solubilization buffer was used as a reference for measuring absorbance. Cut-off values to define rifampicin susceptibility or rifampicin resistance were determined based on a previous report,⁸ and on the RODU values obtained for these four strains.

Screening of strains of M. tuberculosis

Ninety-two coded clinical isolates and H₃₇Rv were screened for rifampicin resistance using the MTT assay as described above. The results were compared with the results obtained with the Bactec method at the end of the study. The bacterial suspensions were checked for contamination by subculturing on blood agar. The results of both the third and sixth day assays of all strains were assessed by the naked eye by comparing the intensity of blue formazan formation in drug containing tubes and controls. Assessment by naked eye was made before measurement of absorbance. Third and sixth day RODU values were also calculated. A strain was defined as rifampicin-suscep-

tible if 1) RODU ≤ 0.2 was obtained at least on the sixth day assay, and 2) a decrease in OD from the third to the sixth day was obtained in rifampicin tubes, while there was an increase in both controls. A strain was defined as resistant if 1) RODU > 0.5 was obtained on both the third and sixth day assays, and 2) an increase in OD from the third to the sixth day was obtained in the controls and the rifampicin-containing tubes. An RODU of between 0.2 and 0.5 on the sixth day assay was defined as borderline resistance. Results obtained by observation by the naked eye and those obtained by measuring absorbance were recorded separately.

RESULTS

The amount of formazan production detected by measuring OD at 570 nm was directly proportional to the initial bacterial concentration and the duration of incubation at 37°C. Figure 1 shows the OD values recorded for some of the bacterial concentrations used to standardise the inoculum size. A 1:100 dilution of the bacterial suspension with turbidity corresponding to the turbidity of McFarland 2 gave a measurable formazan formation on the third day and a grossly visible formazan formation on the sixth day. A bacterial density that corresponded to the turbidity of McFarland 2 was chosen for the MTT assay in this study.

Thirteen strains were identified as rifampicin-resistant, one was identified as borderline rifampicin-

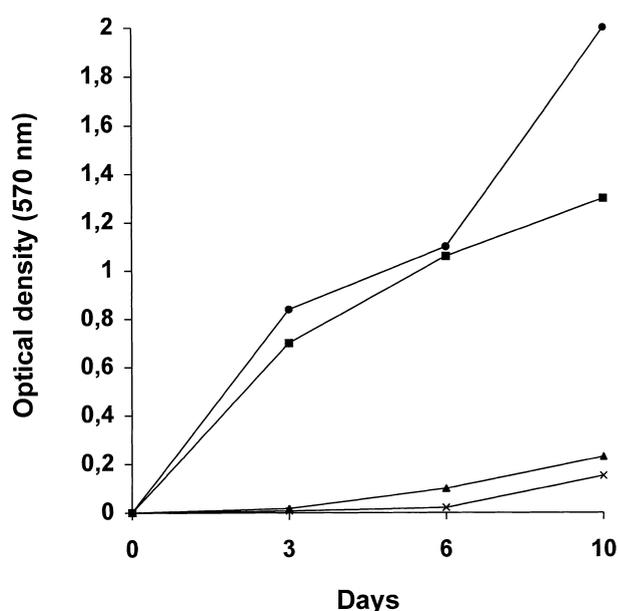


Figure 1 Influence of initial bacterial concentration on the conversion of MTT to blue formazan. Symbols: (●) = 8×10^6 , (■) = 3×10^6 , (▲) = 8×10^4 , (×) = 3×10^4 bacteria per ml. 3×10^4 , which is the 1:100 diluted bacterial suspension of 3×10^6 , was the lowest dilution that gave a measurable OD on the third day assay.

Table RODU values of rifampicin-susceptible and rifampicin-resistant strains of *M. tuberculosis*

	RODU values (Mean \pm SD)	
	Third day	Sixth day
Rifampicin-susceptible ($n = 78$)	0.18 \pm 0.096	0.046 \pm 0.027
Rifampicin-resistant ($n = 13$)	0.94 \pm 0.07	0.99 \pm 0.12
Borderline rifampicin-resistant ($n = 1$)	0.33	0.23

RODU = relative optical density unit.

resistant, and 78 were identified as rifampicin-susceptible by the MTT assay. The results for the susceptible and resistant strains perfectly matched the susceptibility result obtained by the Bactec method. The Table shows the RODU values (mean \pm SD) for the tested strains. The sixth day mean OD value of susceptible strains was 0.089 ± 0.066 , and the mean OD value of the 1:100 diluted controls on the same day was 0.12 ± 0.09 . Fifty-two of the 78 (67%) strains identified as rifampicin-susceptible had OD values less than or equal to those of the 1:100 diluted control. All susceptible strains showed a decrease in OD value between the third and sixth days. The OD values of resistant strains were comparable with those of the undiluted control, giving an RODU of about 1. All strains identified as resistant showed an increase in OD value between the third and sixth days. The third and sixth day RODU values for H₃₇Rv were 0.2 and 0.05, respectively.

One strain (S: 70) had a border-line result with RODU values of 0.33 and 0.23 on the third and sixth days, respectively. This strain also showed an increase in OD values in the rifampicin-containing tube on the sixth day (from OD 0.25 on the third day to 0.5 on the sixth day), suggesting the presence of a significant bacterial population resistant to rifampicin. A repeat MTT test for this strain gave the same result. The minimum inhibitory concentration (MIC) of rifampicin subsequently determined on Bactec was 2 μ g/ml.

The result of the MTT assay was also interpretable by the naked eye. Figure 2 shows typical colour change in a rifampicin-susceptible and two rifampicin-resistant strains. All rifampicin-susceptible and rifampicin-resistant strains were correctly identified by the naked eye on the third and sixth day. The strain (S: 70) with a border-line result was interpreted as susceptible by the naked eye.

DISCUSSION

There is a great need for new mycobacterial diagnostic tests for use in low-income countries. Drug susceptibility testing of *M. tuberculosis* by conventional methods is complicated and time-consuming. Most low-income countries have limited capacity to perform drug susceptibility testing of tubercle bacilli, even for patients suspected of harbouring drug-resistant

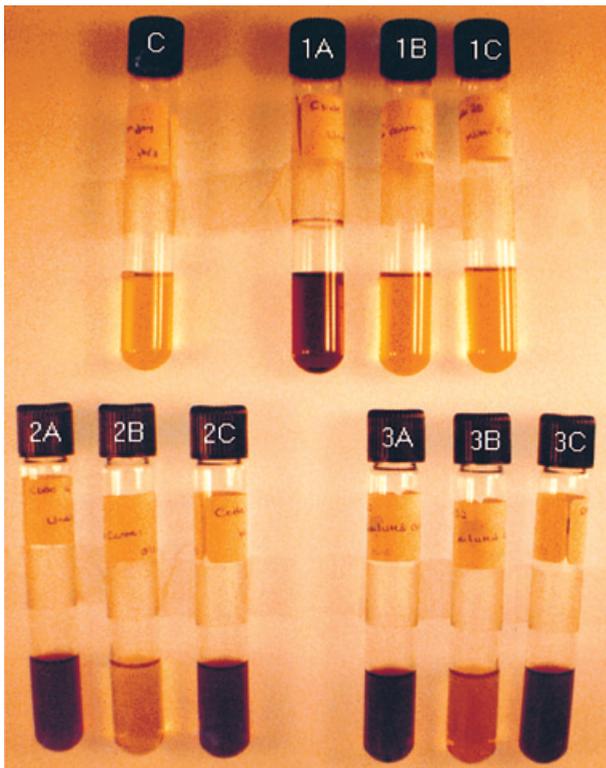


Figure 2 Typical colour changes in the MTT assay with sets of tubes for one rifampicin-susceptible (1A-C) and two rifampicin-resistant strains (2A-C and 3A-C). C = control containing MTT and solubilization buffer but without bacteria; 1–3 A = undiluted bacterial suspension, MTT, and solubilization buffer but no rifampicin; 1–3 B = 1/100 diluted bacterial suspension, MTT, and solubilization buffer but no rifampicin; 1–3 C = undiluted bacterial suspension, MTT, solubilization buffer, and rifampicin 2 µg/ml.

strains. For practical purposes, it is most important to identify patients infected with MDR strains, since these patients will not respond to the re-treatment regimens recommended by the World Health Organization (WHO). Rifampicin resistance is a strong predictor of MDR-TB,¹ and a rapid diagnostic method designed to detect rifampicin resistance is important for a timely modification of treatment and control of the spread of MDR-TB.

The characteristics of the MTT assay performed in 96 well microtiter plates have been described previously.⁸ Using cultures containing various proportions of rifampicin-resistant and rifampicin-susceptible *M. tuberculosis*, it was previously shown that the MTT assay could detect bacterial populations with a drug-resistant sub-population of more than one per cent. However, we found it difficult to standardise the micro-assay for routine use in clinical practice. Since formazan production depends on the number of live cells, the amount of nutrients could be a limiting factor in the micro-assay.¹¹ When the MTT assay was performed in 10 ml tubes, it was possible to use a larger amount of growth media. Moreover, the use of

screw cap tubes reduces the risk of laboratory infection and made the detection of gross colour changes easier. The estimated cost of materials and reagents needed for the MTT assay is about US \$ 0.60 for each *M. tuberculosis* isolate. Except for the borderline resistant strain, all results were also correctly interpreted by the naked eye as early as the third day. Therefore, the cost of the MTT assay could be reduced by half if only the third day MTT assay was performed.

The Bactec system is a proportion method where the growth in drug-containing vials is compared with the growth in the 1:100 diluted control. The Bactec results are interpreted when the growth index (GI) value of the control reaches 30. The difference in GI values of the drug-containing vials on the third and fourth day as compared to the difference in GI values of the control on the same day is used to define drug-susceptible and resistant strains. In the MTT assay, both an undiluted and a 1:100 diluted control were included. Bacterial growth in the drug-containing tube and the diluted control was used as one of the criteria, similar to that used in the Bactec system, to define susceptibility and resistance. The strain identified as borderline resistant (S: 70) by the MTT assay had a MIC of 2 µg/ml as determined by the Bactec method. Strains with an MIC of 2 µg/ml are considered as borderline resistant by the Bactec method.⁴ Thus, there was perfect agreement between the results obtained by the MTT assay and by the Bactec method. The same strain (S: 70) was interpreted as susceptible by the naked eye; this discrepancy is defined as a minor error.¹²

The MTT assay could theoretically be used for the susceptibility testing of all first-line antituberculosis drugs. The assay was tested for the detection of INH-resistance (data not shown). However, the formazan production in tubes with INH was high, and it was not possible to use a cut-off point similar to that used for the detection of rifampicin resistance. It is known that factors that increase the level of reduced pyridine nucleotides and superoxides could increase MTT reduction.^{13,14} Despite an inhibition of mycobacterial proliferation, INH may act in a way that favours MTT reduction.¹⁴ Further standardisation of the MTT assay is needed to enable reliable detection of INH resistance. Combining the MTT assay with other methods such as assay of catalase activity (which could identify strains that are highly resistant to INH) may aid in the rapid detection of MDR strains.

The MTT assay described in the present study should not replace the susceptibility testing intended for surveillance of drug resistance in national tuberculosis programmes. This surveillance should be routinely carried out at national level, using conventional methods. However, the MTT assay could be used as a simple and rapid method to identify patients infected with MDR-TB strains. It could also potentially be

used as a simple screening method for the study of new anti-mycobacterial drugs.

Acknowledgements

This work was supported by a research grant from the Danish National Association Against Lung Diseases.

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RÉSUMÉ

OBJECTIFS : Standardisation du test colorimétrique basé sur le 3-(4,5-diméthylthiazol-2-yl)-2,5-diphényl bromure de tétrazolium (MTT) pour la détection rapide de la résistance de *Mycobacterium tuberculosis* à la rifampicine en pratique clinique, et évaluer le test sur un collectif de 92 isolats cliniques.

SCHÉMA : La méthode Bactec a été utilisée comme méthode de référence. La rifampicine, à la concentration de 2 µg/ml, a été utilisée pour tester la sensibilité par la technique Bactec. Le test MTT a été conduit dans des tubes contenant 3 ml de milieu de Dubos. Le test est basé sur le principe que les cellules vivantes convertissent le sel jaune de tétrazolium en formazan bleu. Une concentration finale de 2 µg/ml de rifampicine est étudiée dans le test. Des valeurs de densité optique (OD) à 570 nm sont enregistrées le 3^e et le 6^e jour. Une souche est considérée comme sensible lorsque l'unité de densité optique relative (RODU) (c'est-à-dire la densité

optique du tube contenant la rifampicine sur la densité optique du contrôle non dilué) est égale ou inférieure à 0,2, et lorsque la valeur de la densité optique du tube contenant la rifampicine au 6^e jour est inférieure à la valeur de la densité optique au 3^e jour. Une souche est considérée comme résistante lorsque le RODU est supérieur à 0,5, et lorsque la valeur de la densité optique dans le tube contenant la rifampicine augmente au 6^e jour. Une lecture visuelle directe des tubes a également été conduite.

RÉSULTATS ET CONCLUSION : Les résultats obtenus avec le test MTT concordent parfaitement avec ceux obtenus par la méthode Bactec. Le test MTT est également interprétable à l'oeil nu. Ce test simple et peu coûteux pourrait être utilisé comme méthode rapide de dépistage pour l'identification des souches résistantes à la rifampicine dans les pays à faibles revenus.

RESUMEN

OBJETIVOS : Estandarizar el test colorimétrico basado en 3-(4,5-dimetiltiazol-2-yl)-2,5-bromuro de difenil tetrazolium (MTT) para la detección rápida de la resistencia del *Mycobacterium tuberculosis* a la rifampicina en la práctica clínica y evaluar el test en 92 muestras de material.

MÉTODO : Se utilizó el Bactec como método de refe-

rencia. Se usó la rifampicina, a una concentración de 2 µg/ml, para el test de sensibilidad con el método Bactec. El test MTT se efectuó en tubos que contenían 3 ml de caldo de Dubos. El test está basado en el principio de que las células vivas convierten el tetrazolium amarillo en formazan azul. En el ensayo se utilizó una concentración final de 2 µg/ml de rifampicina. Se re-

gistraron valores de densidad óptica (DO) de 570 nm al tercer y sexto día. Se definió una cepa como sensible cuando la unidad de densidad óptica relativa (UDOR) (o sea, DO de rifampicina del tubo/DO del control no diluido) era 0,2 y cuando el valor DO del tubo con rifampicina al sexto día era menor que el valor DO del tercer día. Se definió una cepa como resistente cuando el UDOR era mayor de 0,5 y cuando había un aumento en el valor DO del tubo con rifampicina al

sexto día. Los tubos fueron también visualmente observados.

RESULTADOS Y CONCLUSIÓN: Los resultados obtenidos con el MTT coinciden perfectamente con aquellos obtenidos con el método Bactec. El test MTT también puede ser interpretado visualmente. Este test simple y barato puede ser utilizado para identificar las cepas resistentes a la rifampicina en los países con pocos recursos.
