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Comparative evaluation of the nitrate reduction assay, the MTT test, and the resazurin microtitre assay for drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis*

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Objectives: To evaluate the performance of three rapid low-cost methods for the detection of resistance to first-line drugs in *Mycobacterium tuberculosis*.

Methods: One hundred *M. tuberculosis* clinical isolates were tested by the nitrate reductase assay (NRA), the MTT test and the resazurin microtitre assay (REMA), and the results compared with those obtained with the gold standard proportion method (PM) on Löwenstein Jensen medium.

Results: The results using the three methods showed a good sensitivity and specificity between 94% and 100% for the detection of rifampicin and isoniazid resistance. Specificity for ethambutol and streptomycin using MTT and resazurin was low (58–89%). In contrast, NRA showed a good agreement for all first-line drugs tested.

Conclusions: This study shows a high level of agreement of these three low-cost methods compared with the PM for rapid detection of rifampicin and isoniazid resistance. However, more standardization is needed for ethambutol and streptomycin using the MTT test and resazurin microtitre assay. The nitrate reductase assay might represent an inexpensive procedure for rapid detection of resistance to first-line drugs in low-resource countries.

Keywords: antibiotic resistance, colorimetric, rapid methods

Introduction

Tuberculosis (TB) is still an infectious disease with a high morbidity and mortality around the world. According to the latest World Health Organization (WHO) report, there were 8.8 million new TB cases in 2002 and more than two million deaths were attributed to the disease.[1] Moreover, the worldwide emergence of multidrug-resistant (MDR) TB is a compounding problem as has been recently reported.[2] Thus, rapid and reliable methods for diagnosis and drug susceptibility testing (DST) in TB are urgently needed. The conventional methods for DST of *Mycobacterium tuberculosis* include the proportion method (PM) on Löwenstein Jensen (LJ) medium or Middlebrook 7H10 agar[3] and the radiometric method in the BACTEC TB-460 system[4] (Becton Dickinson). However, they require either several weeks to give results or depend on heavily mechanized equipment that uses radioactive compounds.

In the last few years, several methods have been proposed for the rapid detection of drug resistance and for DST of *M. tuberculosis*.[5–8] Among them, the Mycobacterial Growth Indicator Tube (MGIT, Becton Dickinson), and molecular tools such as the INNO-LiPA Rif.TB (Line probe assay, Innogenetics, Ghent, Belgium) have extensively been applied. However, they are expensive and impractical for routine use. Ängeby *et al.* have described a new nitrate reductase assay (NRA) for rapid and inexpensive DST of *M. tuberculosis*.[9] The method depends on the ability of *M. tuberculosis* to reduce nitrate to nitrite which can be detected using specific reagents producing a change in colour. Abate and colleagues have also proposed a rapid colorimetric method based on the reduction of the MTT

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compound for detection of resistance to rifampicin.[10,11] More recently, Morcillo *et al.* have used this same compound for determining the susceptibility of MDR *M. tuberculosis* strains to several second-line anti-TB drugs in a microplate indicator-based method.[12]

We have been involved in the development of a rapid micro-plate method, the Resazurin Microtitre Assay (REMA) plate for the detection of MDR strains of *M. tuberculosis*.[13] The method was successfully tested on clinical isolates against isoniazid and rifampicin with results obtained after 7 days. More recently, Martin *et al.* have applied this same method for DST of clinical isolates of *M. tuberculosis* against second-line anti-TB drugs with very good results.[14]

In this study, we have performed for the first time a comparative evaluation of the nitrate reductase assay, the MTT reduction test and the REMA plate for DST of 100 clinical isolates of *M. tuberculosis* to first-line drugs. The results obtained were compared with those of the PM on LJ medium.

Materials and methods

Mycobacterial isolates

One hundred clinical isolates of *M. tuberculosis* from the collection of the reference laboratory of the Instituto de Medicina Tropical 'Pedro Kouri', Havana, Cuba, were evaluated. Reference strains H37Rv (ATCC 27294), and rifampicin-resistant (ATCC 35838), isoniazid-resistant (ATCC 35822), ethambutol-resistant (ATCC 35837) and streptomycin-resistant (ATCC 35820) strains from the American Type Culture Collection, were used as susceptible and resistant controls. All strains were freshly sub-cultured on LJ medium before being used.

Anti-tuberculous drugs

Rifampicin, isoniazid, ethambutol and streptomycin were obtained as powder from Sigma–Aldrich (Bornem, Belgium). The stock solutions were prepared in advance at a concentration of 10 g/L in methanol for rifampicin, and 1 g/L in distilled water for the other drugs. Stock solutions were filter sterilized and kept at $-20\,°C$ for not more than 1 month. Working solutions of each drug were prepared at four-fold the highest concentration tested on the plates.

Drug susceptibility testing

The PM was carried out on LJ medium according to the standard procedures with the recommended critical concentrations of 40 mg/L for rifampicin, 0.2 mg/L for isoniazid, 2 mg/L for ethambutol and 4 mg/L for streptomycin.[15]

Nitrate reductase assay (NRA)

This method is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, which is routinely used for biochemical identification of mycobacterial species. The presence of nitrite can easily be detected with specific reagents, which produce a change in colour. The nitrate reductase assay uses the detection of nitrite as an indication of growth when used as a drug susceptibility test. The antibiotic was included in the LJ medium at a concentration of 40 mg/L for rifampicin, 0.2 mg/L for isoniazid, 2 mg/L for ethambutol and 4 mg/L for streptomycin; 1000 mg/L of potassium nitrate (KNO_3) was also added. The inoculum was adjusted to a turbidity equivalent to that of a no. 1 McFarland Standard, and diluted 1:10

in phosphate buffered saline (PBS). The reagent mix consisted of 1 part 50% concentrated hydrochloric acid (HCl), 2 parts 0.2% sulfanilamide, and 2 parts 0.1% *N*-1-naphthylethylenediamine dihydrochloride. The method was carried out as described by Ängeby *et al.*[9] For each strain, 200 μL of the undiluted inoculum was added to the antibiotic tube, and 200 μL of the 1:10 dilution to the drug-free tube as a growth control. The tubes were incubated at 37 °C. After 7 days, 500 μL of the reagent mixture was added to one drug-free tube. If any colour change appeared, all the tubes were developed with the reagent mixture; otherwise, the tubes were re-incubated and the procedure repeated at day 10 and day 14. An isolate was considered resistant if colour developed in the test tube (pink to red or purple) and this colour was greater than that appearing in the 1:10-diluted growth control.

Reagents for colorimetric methods

A stock solution of resazurin sodium salt powder (Acros Organic N.V., Geel, Belgium) was prepared at 0.01% in distilled water, filter sterilized and kept at 4 °C.

A stock solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma–Aldrich, Belgium) at a concentration of 5 g/L was prepared in PBS, pH 6.8, and kept at 4 °C in the dark. Formazan solubilization buffer was prepared by mixing 1:1 (v/v) 20% sodium dodecyl sulphate (SDS) and a solution of 50% *N*,*N*-dimethylformamide (DMF).

REMA

The REMA plate method was carried out as described by Palomino *et al.*[13] Briefly, the inoculum was prepared from a fresh LJ medium in 7H9-S broth, adjusted to a McFarland tube No.1 and diluted 1:20; 100 μL was used as the inoculum. One hundred microlitre volumes of 7H9-S broth were dispensed in each well of a sterile 96-well flat bottom plate (Becton Dickinson) and serial two-fold dilutions of each drug were prepared directly on the plate by adding 100 μL of the working solution of each drug to achieve the final concentrations. The range of concentrations tested were 0.062–2.0 mg/L for rifampicin, 0.031–1.0 mg/L for isoniazid, 1–32 mg/L for ethambutol, and 0.25–8 mg/L for streptomycin. Then, 100 μL of the inoculum was added to each well. A growth control containing no antibiotic and a sterile control without inoculum were also included for each isolate. Two hundred microlitres of sterile water was added to all perimeter wells to avoid evaporation during the incubation. The plates were covered and replaced in their original plastic bags and incubated at 37 °C in a normal atmosphere. After 7 days of incubation, 30 μL of resazurin solution was added to each well and the plate was re-incubated overnight. A change in colour from blue (oxidized state) to pink (reduced) indicated the growth of bacteria and the minimal inhibitory concentration (MIC) was defined as the lowest concentration of each drug that prevented this change in colour.

MTT assay

This method was carried out as described by Abate *et al.*[10] The inoculum was prepared as described above for the REMA plate method and the drug concentration ranges used were the same. Preparation of the 96-well plates was identical as described for the REMA plate. After 7 days of incubation at 37 °C, 10 μL of the MTT solution (5 g/L) was added to each well and the plate was re-incubated overnight. If a violet precipitate (formazan) appeared, 50 μL of the SDS/DMF solution was added to the wells and the plate re-incubated for 3 h. A change in colour from yellow to violet

indicated growth of bacteria and the MIC was interpreted as in the REMA plate.

Data analysis

Analysis of data was carried out with MedCalc software (MedCalc, Belgium). Receiver operating characteristic (ROC) curve analysis was also carried out with MedCalc.

Results

One hundred clinical isolates of *M. tuberculosis* were analysed by the NRA, MTT, REMA and PM methods. Table 1 shows the results obtained with NRA compared to PM using LJ as the gold standard. For isoniazid, 43 isolates were found resistant and 55 susceptible by both methods. Two isolates gave discordant results since they were resistant by PM but susceptible by NRA. For rifampicin, all 100 isolates gave concordant results with 37 resistant and 63 susceptible isolates. For ethambutol, 17 isolates were resistant and 82 susceptible by both methods; one strain gave a discordant result being susceptible by PM but resistant by NRA. For streptomycin, 45 isolates were resistant and 51 susceptible by both methods. One isolate was susceptible by PM but resistant by NRA, and three isolates were resistant by PM but susceptible by NRA. Sensitivity values for isoniazid, rifampicin, ethambutol and streptomycin were 95.6%, 100%, 100% and 93.7%, respectively and specificity values were 100%,

Table 1. Susceptibility testing of 100 clinical isolates of *M. tuberculosis* by the nitrate reductase assay and the proportion method

	Proportion method							
	INH		RIF		EMB		STR	
NRA	R	S	R	S	R	S	R	S
Resistant	43	0	37	0	17	1	45	1
Susceptible	2	55	0	63	0	82	3	51
Total	45	55	37	63	17	83	48	52

NRA, nitrate reduction assay; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; STR, streptomycin; R, resistant; S, susceptible.

Table 2. Susceptibility testing of 100 clinical isolates of *M. tuberculosis* by the MTT test and the proportion method

	Proportion method							
	INH		RIF		EMB		STR	
MTT	R	S	R	S	R	S	R	S
Resistant	45	2	37	0	16	24	44	6
Susceptible	0	53	0	63	1	59	4	46
Total	45	55	37	63	17	83	48	52

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; STR, resistant; S, susceptible.

Table 3. Susceptibility testing of 100 clinical isolates of *M. tuberculosis* by REMA and the proportion method

	Proportion method							
	INH		RIF		EMB		STR	
REMA	R	S	R	S	R	S	R	S
Resistant	45	2	37	1	16	35	45	6
Susceptible	0	53	0	62	1	48	3	46
Total	45	55	37	63	17	83	48	52

REMA, resazurin microtitre assay; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; STR, streptomycin; R, resistant; S, susceptible.

100%, 98.7% and 98.0%, respectively. The overall concordance was 98.2%.

The results obtained with MTT assay are shown in Table 2. Cut-off values determined with MedCalc software were 0.25, 0.25, 4.0 and 1.0 mg/L for isoniazid, rifampicin, ethambutol, and streptomycin, respectively. For isoniazid, 45 isolates were resistant and 53 susceptible by both methods; two isolates were found susceptible by the PM but resistant by MTT. For rifampicin, all results were concordant with 37 resistant and 63 susceptible isolates. For ethambutol, 16 isolates were resistant and 59 susceptible by both methods; 25 isolates gave discordant results with 24 of them susceptible by the PM but resistant by MTT and one isolate resistant by the PM but susceptible by MTT. For streptomycin, 44 isolates were resistant and 46 susceptible by both methods; 10 isolates gave discordant results with six of them susceptible by the PM but resistant by MTT and four isolates resistant by the PM but susceptible by MTT. Sensitivity values for isoniazid, rifampicin, ethambutol and streptomycin were 100%, 100%, 94.1% and 91.7%, respectively and specificity values of 96.4%, 100%, 71.1% and 88.5%, respectively were obtained. The overall concordance was 90.7%.

For the REMA test, the results obtained are shown in Table 3. The same cut-off values were obtained as for the MTT test. For isoniazid, 45 isolates were found resistant and 53 susceptible by both methods; two isolates were susceptible by the PM but resistant by REMA. For rifampicin, 37 isolates were resistant and 62 susceptible by both methods; one isolate was susceptible by the PM but resistant by REMA. For ethambutol, 16 isolates were found resistant and 48 susceptible by both methods; 35 isolates were susceptible by the PM but resistant by REMA and one isolate was resistant by the PM but susceptible by REMA. For streptomycin, 45 isolates were resistant and 46 susceptible by both methods; six isolates were susceptible by the PM but resistant by REMA and three isolates were resistant by the PM but susceptible by REMA. The sensitivity for isoniazid, rifampicin, ethambutol and streptomycin were 100%, 100%, 94.1% and 93.8%, respectively and the specificity 96.4%, 98.4%, 57.8% and 88.5%, respectively. The overall concordance was 88.2%.

In order to assess in more detail the performance of the MTT, REMA and NRA tests, ROC curve analysis was carried out with MedCalc software. Figures 1, 2 and 3 show the ROC curves for MTT, REMA and NRA, respectively. Area under the curve (AUC) values obtained for isoniazid, rifampicin, ethambutol and streptomycin were, respectively, 0.998, 1.0, 0.868, and 0.973
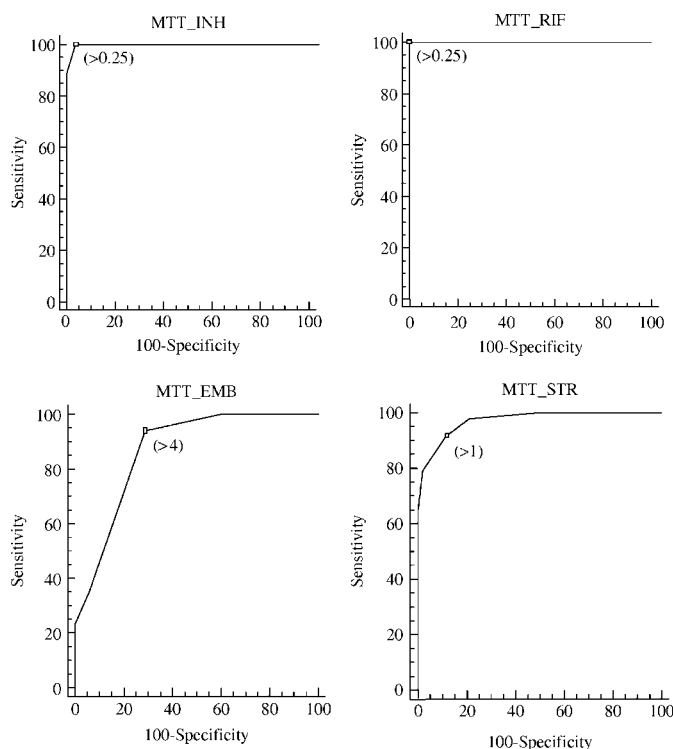
Figure 1. ROC curve analysis for MTT assay. Area under ROC curve is 0.998 for isoniazid (INH), 1.000 for rifampicin (RIF), 0.868 for ethambutol (EMB) and 0.973 for streptomycin (STR).
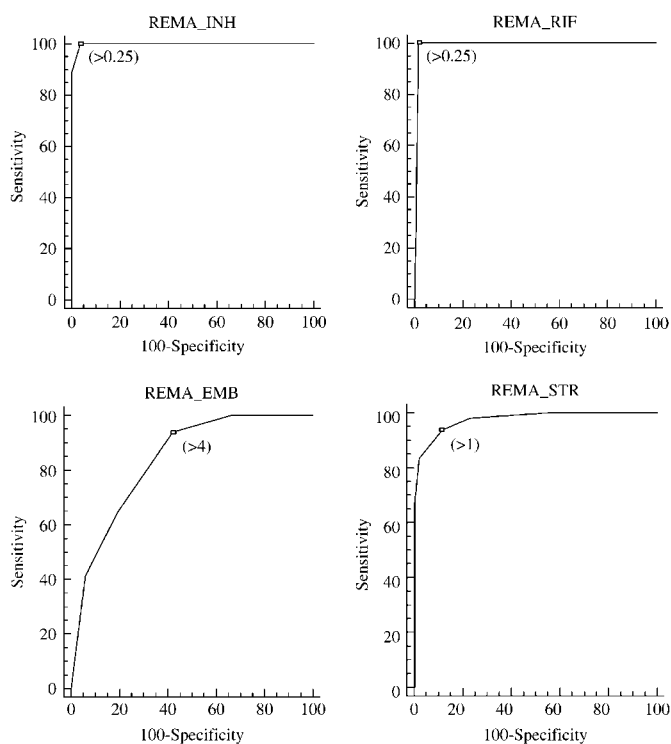

Figure 2. ROC curve analysis for REMA. Area under ROC curve is 0.991 for isoniazid (INH), 0.992 for rifampicin (RIF), 0.832 for ethambutol (EMB) and 0.976 for streptomycin (STR).

with MTT, 0.991, 0.992, 0.832 and 0.976 with REMA, and 0.978, 1.0, 0.994 and 0.959 with NRA. None of these values was statistically significantly different showing comparable results for all tests as can be observed in Figure 4 for each drug.

Discussion

Traditional drug susceptibility testing such as the PM on LJ or agar medium is time consuming. The BACTEC radiometric system has the advantage of being more rapid than the proportion method (5–10 days) but it requires the use of radioisotopes and can be costly to perform.[4] Other commercial tests[16] and molecular tools (INNO-LiPA) have been developed but are very expensive and impractical for routine use.[4,17] For developing countries, it would be helpful to have a simple and inexpensive test that can rapidly detect resistant *M. tuberculosis* strains. The contribution of TB laboratories worldwide (through rapid and accurate susceptibility testing) is very important for the management of MDR TB, especially in low-resource countries where most cases of MDR TB occur. The results obtained with the colorimetric methods MTT, resazurin and nitrate reductase assays, were available on an average of 10 days as with the BACTEC TB-460 system. Each method has been successfully tested in previous studies,[8,13,18–20] but never compared together against the first-line anti-tuberculosis drugs. In this study, MTT and REMA gave the same results and the area under the ROC curve showed no significant differences between them.

For rifampicin and isoniazid, both methods showed 100% sensitivity and more than 96% specificity. These results are very important since rifampicin and isoniazid are the two most important drugs used in the treatment of TB. Results for ethambutol and streptomycin, two drugs known to be difficult to test, showed a sensitivity higher than 92% but the specificity was low
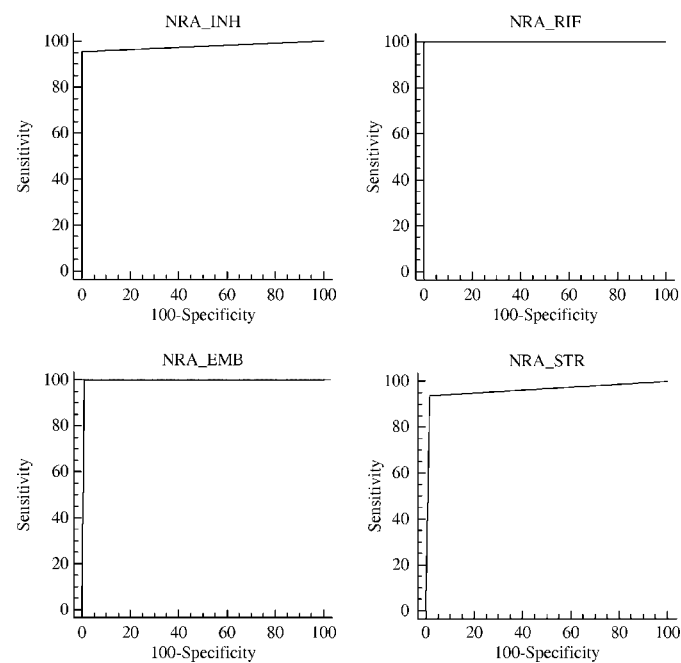

Figure 3. ROC curve analysis for NRA. Area under ROC curve is 0.978 for isoniazid (INH), 1.0 for rifampicin (RIF), 0.994 for ethambutol (EMB) and 0.959 for streptomycin (STR).
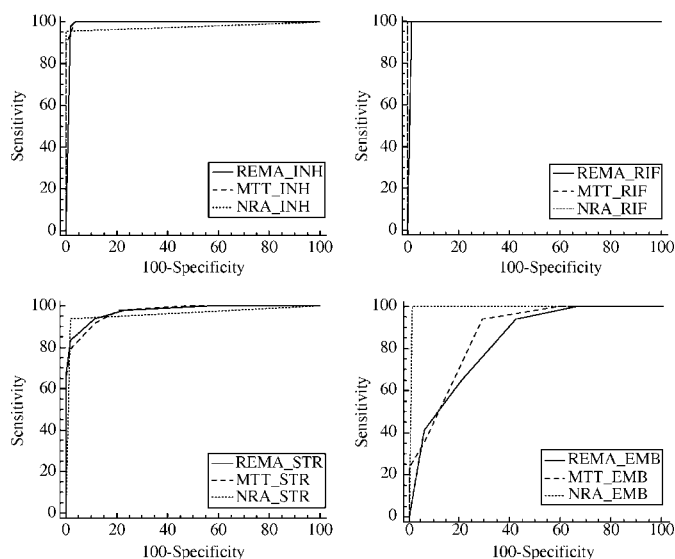
Figure 4. ROC curves of REMA, MTT and nitrate reductase assay. Area under ROC curve difference values for REMA versus MTT, REMA versus NRA and MTT versus NRA were, respectively 0.007, 0.013 and 0.020 for isoniazid (INH), 0.008, 0.008 and 0.000 for rifampicin (RIF), 0.003, 0.017 and 0.014 for streptomycin (STR), and 0.035, 0.162 and 0.126 for ethambutol (EMB).

(58−89%). On the other hand, using the NRA, very good sensitivity and specificity (94−100%) were found for all drugs. Thus, solid medium seems to give more reliable results for ethambutol and streptomycin. This could be one possible explanation for the high number of discordant results obtained with ethambutol and streptomycin where the antibiotics might have deteriorated faster in the liquid medium, especially in the case of ethambutol. More studies are needed, however, to assess the discordant results obtained with streptomycin.

In summary, this study shows that using these three low cost methods for rapid detection of rifampicin and isoniazid resistance, a high level of agreement with the PM was obtained. These new alternative methods seem to have the potential to provide rapid detection of resistance to isoniazid and rifampicin, do not need any sophisticated equipment, are simple to perform, reduce the time to report first results compared to classical conventional methods and could be implemented in laboratories with limited resources. NRA seems to be an inexpensive alternative method for rapid and accurate detection of resistance to all four first-line drugs and has the advantage of being performed on the classical LJ media. MTT and REMA need more standardization for ethambutol and streptomycin and due to their performance in liquid media, MTT and resazurin assays have the disadvantage from the point of view of biosafety that manipulation of plates could generate aerosols. A clinical trial of these new rapid methods is warranted to analyse the possibility of implementing them in low-resource countries.

Acknowledgements

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