

Rapid alternative methods for detection of rifampicin resistance in *Mycobacterium tuberculosis*

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Received 17 February 2004; returned 27 April 2004; revised 4 May 2004; accepted 10 May 2004

Objective: To study the performance of three rapid low cost methods for the detection of rifampicin resistance.

Methods: A panel of 20 coded *Mycobacterium tuberculosis* strains was tested blindly by the low cost methods: nitrate reductase, MTT and resazurin assays, and compared with the results obtained with the gold standard methods: the proportion method on Löwenstein–Jensen medium and the BACTEC TB 460 system. We have also tested two commercial tests: MGIT and INNO LiPA Rif.TB kit.

Results: Complete agreement was observed among all methods.

Conclusion: These three simple methods might become inexpensive alternative procedures for rapid detection of rifampicin resistance in low-resource countries.

Keywords: *M. tuberculosis*, MTT, resazurin, nitrate reductase assay, rifampicin

Introduction

The spread of multidrug-resistant tuberculosis (MDR TB) in the world remains a major public health problem. Early diagnosis of tuberculosis and rapid detection of drug resistance is an urgent priority to identify patients who are not responding to the standard treatment and to avoid dissemination of resistant strains. Rifampicin, discovered in 1963, is the most powerful bactericidal drug against tuberculosis, the most potent sterilizing drug available and a key component for treatment of tuberculosis.¹ Since rifampicin resistance is considered as a surrogate marker for the identification of MDR TB,^{2,3} it would be helpful for low-resource countries to have simple and inexpensive tests than can rapidly detect resistance to rifampicin. The commonly used proportion method (PM) for mycobacterial drug susceptibility testing requires several weeks of incubation to give results.^{4–6} The BACTEC radiometric system (Becton Dickinson, Sparks, MD, USA) has the advantage of being more rapid than the proportion method but requires the use of radioisotopes and can be costly to perform.^{7,8} Other commercial tests such as the MGIT (mycobacterial growth indicator tube) and molecular tools such as the INNO-LiPA Rif.TB (line probe assay; Innogenetics, Ghent, Belgium) have been developed but are also expensive and impractical for routine use.^{9–11} New simple, rapid and inexpensive tests for detection of rifampicin resistance have been recently

developed. The colorimetric methods employing the oxidation–reduction indicator resazurin and MTT have been successfully used to determine minimum inhibitory concentrations (MICs) of rifampicin and several other antituberculosis drugs, a change in colour indicating the growth of bacteria.^{12–19} Another recently described method is the nitrate reductase assay on Löwenstein–Jensen (LJ) based on the ability of *Mycobacterium tuberculosis* to reduce nitrate to nitrite. The presence of nitrite can easily be detected with specific reagents that produce a change in colour.²⁰

The purpose of our study was to evaluate the performance of three rapid low cost methods for the detection of rifampicin resistance: resazurin, MTT and nitrate reductase assays. We compared the results with those obtained by the gold standard methods: the PM on LJ medium and the BACTEC 460 system, with a panel of 20 coded *M. tuberculosis* strains tested in a blind manner to avoid influencing the results of the different methods. In parallel, we have tested this same panel by the MGIT system and determined the *rpoB* mutations with the INNO-LiPA Rif.TB kit.

Materials and methods

Bacterial strains

A panel of 20 clinical isolates of *M. tuberculosis* originating from Peru with known drug susceptibility patterns was studied. Ten were

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MDR and 10 were susceptible strains. Reference strains H37Rv (ATCC number 27294) and a rifampicin-resistant strain (ATCC number 35838), from the American Type Culture Collection, were used as the reference susceptible and resistant controls, respectively. All strains were freshly subcultured on LJ medium before use and tested in a blind manner for all different methods.

Antituberculous drug

Rifampicin was obtained as powder from Sigma–Aldrich (Bornem, Belgium). The stock solution was prepared in advance at a concentration of 10 g/L in methanol, filter sterilized and kept at -20°C for not more than 1 month.

Drug susceptibility testing

The PM was carried out on LJ medium according to the standard procedure with the recommended critical concentration of rifampicin 40 mg/L.^{4,5}

BACTEC 460 system

The standard protocol was followed according to the manufacturer's instructions with a BACTEC 460 instrument (Becton Dickinson, Sparks, MD, USA) using a final rifampicin concentration of 2 mg/L. A loopful of *M. tuberculosis* from fresh LJ medium was transferred to a tube containing several glass beads. The turbidity of the bacterial suspension was adjusted to McFarland tube no. 0.5 in distilled water. To determine the 1% proportion of resistance, the inoculum used in the control vial was 100 less than that used for the drug containing vials. All vials were tested daily after inoculation and incubation at 37°C . The rate of increase in the growth index (GI) or the amount of change over that of the previous day, called ΔGI , was compared for the control vial and the vial containing the rifampicin. If the daily ΔGI in the drug vial was greater than that in the control vial, the strain was considered to be resistant to rifampicin, and if the ΔGI was inferior, the strain was considered to be susceptible.

MGIT

The MGIT test was carried out as described by Palomino *et al.*⁹ Briefly, MGIT tubes were supplemented with 0.5 mL of oleic acid/albumin/dextrose/catalase (OADC), and rifampicin was added to obtain a final concentration of 1 mg/L. A growth control tube was prepared without antibiotic. The inoculum was adjusted to McFarland 0.5 and further diluted 1:5 with sterile saline. The tubes were inoculated with 0.5 mL of the inoculum diluted 1:5. A positive control tube was prepared by adding 5 mL of a 0.4% sodium sulphite solution to an empty MGIT tube and an uninoculated MGIT tube was used as a negative control. After 3 days of incubation at 37°C , the fluorescence of the MGIT tubes was read on a 365 nm UV transilluminator. The growth control tube was compared to the positive and negative controls. Positivity was indicated by bright orange fluorescence at the bottom of the tube and an orange reflection at the meniscus. Negative tubes showed very little or no fluorescence. When fluorescence appeared in the growth control tube, it was considered as day 0 for the interpretation of the drug-containing tube. A strain was considered as susceptible if the drug tube did not fluoresce within 2 days of the positivity of the growth control and as resistant if the drug tube was positive within 2 days of the positivity of the growth control.

INNO LiPA Rif.TB assay

The commercial INNO LiPA Rif.TB kit (Innogenetics, Ghent, Belgium) was used according to the instructions of the

manufacturer. A full loop of bacteria was resuspended in 400 μL of TE buffer, heated at 95°C for 5 min, followed by a short centrifugation at 3000g. Then, 2 μL of the supernatant was transferred to the PCR amplification mixture. *M. tuberculosis* strain H37Rv was used as a control. The *rpoB* gene was amplified with specific biotin-labelled primers by using 1 U of *Taq* DNA polymerase per reaction mixture in a thermocycler. The amplification was checked by 2% agarose gel electrophoresis. The amplicon appeared as a single band with a length of 260 bp. The PCR product was then denatured and hybridized to a strip with 10 specific oligonucleotide probes. The reactivities of an amplified fragment with the S-type probes for the wild-type (probes S1 through S5) were used to detect the mutations that lead to rifampicin resistance in *M. tuberculosis*. Four probes (R-type probes) are specifically designed to hybridize to the sequences of the four most frequently observed mutations: R2 (Asp-516 \rightarrow Val), R4a (His-526 \rightarrow Tyr), R4b (His-526 \rightarrow Asp) and R5 (Ser-531 \rightarrow Leu). When all the wild-type S probes gave a positive signal and all the R probes reacted negatively, the *M. tuberculosis* isolate was considered susceptible to rifampicin. When at least one negative signal was obtained with the wild-type S probes, the isolate was considered rifampicin-resistant (Inno-LiPA Rif.TB S patterns).

Colorimetric method reagents

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 was kept at 4°C in the dark. Formazan solubilization buffer was prepared by mixing 1:1 (v/v) 20% SDS and a solution of 50% *N,N*-dimethylformamide (DMF).

Resazurin microtitre assay

The resazurin microtitre assay (REMA) plate method was carried out as described by Palomino *et al.*¹⁵ Briefly, the inoculum was prepared from fresh LJ medium in 7H9-S medium, adjusted to a McFarland tube no. 1, then diluted 1:20 and 100 μL was used as an inoculum. One hundred microlitres of 7H9-S broth was dispensed in each well of the sterile 96-well plate (flat bottom; Becton Dickinson) and serial two-fold dilutions of rifampicin were prepared directly on the plate by adding 100 μL of the working solution of drug to achieve the final concentration. The rifampicin concentration range used was 0.062–2.0 mg/L. Then, 100 μL of inoculum was added to each well. A growth control well containing no drug and a sterile control 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 plate was covered, sealed in a plastic bag 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 reincubated overnight. A change in colour from blue (oxidized state) to pink (reduced) indicated the growth of bacteria and the MIC was defined as the lowest concentration of drug that prevented this change in colour.

MTT assay

This method was carried out as described by Abate *et al.*¹² The inoculum was prepared as described above for the REMA plate method and the rifampicin concentration range used was also the same (0.062–2.0 mg/L). The procedure to prepare the 96-well plate was identical to the REMA plate assay. 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 reincubated overnight. If a violet precipitate (formazan) appeared in the MTT well, 50 µL of the SDS/DMF solution was added to these wells and the plate reincubated for 3 h. A change in colour from yellow to violet indicated the growth of bacteria and the MIC was interpreted as in the REMA plate assay.

Nitrate reduction 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 colour change. The nitrate reduction assay uses the detection of nitrite as an indication of growth when used as a drug susceptibility test. The rifampicin was included in the LJ medium at a concentration of 40 mg/L with 1000 mg/L potassium nitrate (KNO₃). The inoculum turbidity was adjusted to a McFarland tube no. 1 and diluted 1:10 in PBS. The reagent mixture 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 *Ángeby et al.*²⁰ For each strain, 200 µL of the undiluted suspension was inoculated into the antibiotic tube, and 200 µL of the 1:10 dilution into the drug-free tube. The tubes were incubated at 37°C. After 7 days, 500 µL of reagent mixture was added to one drug-free tube. If any colour occurred, all the tubes were developed by the reagent mixture, otherwise the tubes were reincubated and the procedure was repeated at day 10 and day 14. A strain was considered resistant if there was a colour change in the antibiotic tube greater than the 1:10-diluted growth control.

Results

Results are presented in Table 1. Complete agreement in results was found between the three different methods studied compared to those obtained by the PM. With the BACTEC 460 system, it was possible to obtain results in 6–13 days and by the MGIT system results were obtained between 4 and 13 days. Ten strains were defined as resistant to rifampicin and 10 strains as susceptible. With the commercial kit INNO LiPA Rif.TB, 10 strains showed a susceptible pattern to rifampicin and 10 a resistant pattern. These results were obtained in 1 day and were the same as obtained by the BACTEC and MGIT system. For the colorimetric methods, by visual reading of the assay plate, the MIC of rifampicin for all isolates was obtained in a period of 8–13 days both for MTT and resazurin. For both methods, 10 strains were found resistant and 10 susceptible. For the susceptible strains, MIC values were <0.062 mg/L. One of the 10 resistant strains showed an MIC of 1 mg/L by the MTT assay and for all the other strains the MIC was >2.0 mg/L. The reading for the nitrate reductase assay was obtained from 7 to 14 days but the majority of the results were available at 10 days. Complete agreement was found in results for rifampicin when compared with the PM.

Discussion

The results obtained in this study are very important since rifampicin resistance is considered as a surrogate marker of MDR, especially in countries with a high prevalence of drug

Table 1. Results of rifampicin resistance of a panel of *M. tuberculosis* strains by the different techniques

Strain no.	PM	BACTEC 460	MGIT	Nitrate	LiPA		MIC colorimetric (mg/L)	
					pattern	result	MTT	resazurin
1	R	R	R	R	R2	R	>2.0	>2.0
2	S	S	S	S	WT	S	<0.062	<0.062
3	S	S	S	S	WT	S	<0.062	<0.062
4	R	R	R	R	R5	R	>2.0	>2.0
5	S	S	S	S	WT	S	<0.062	<0.062
6	R	R	R	R	DS1	R	>2.0	>2.0
7	R	R	R	R	R2	R	>2.0	>2.0
8	S	S	S	S	WT	S	<0.062	<0.062
9	S	S	S	S	WT	S	<0.062	<0.062
10	R	R	R	R	R5	R	>2.0	>2.0
11	R	R	R	R	R2	R	>2.0	>2.0
12	S	S	S	S	WT	S	<0.062	<0.062
13	S	S	S	S	WT	S	<0.062	<0.062
14	R	R	R	R	R5	R	>2.0	>2.0
15	S	S	S	S	WT	S	<0.062	<0.062
16	R	R	R	R	DS1	R	>2.0	>2.0
17	R	R	R	R	R2	R	1	2
18	S	S	S	S	WT	S	<0.062	<0.062
19	S	S	S	S	WT	S	<0.062	<0.062
20	R	R	R	R	R5	R	>2.0	>2.0
H37Rv	S	S	S	S	WT	S	<0.062	<0.062
RIF R	R	R	R	R	R5	R	>2.0	>2.0
TAT (days)	28–42	6–13	4–13	7–14		1–2	8–13	8–13

TAT, turnaround time (range); WT, wild-type; R2, mutation Asp-516 → Val; ΔS1, mutation in probe region 1; R5, mutation Ser-531 → Leu.

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resistance.³ Traditional drug susceptibility testing such as the PM on LJ and the BACTEC 460 system are time consuming. The development of rapid and inexpensive new methods for resistance detection is an urgent priority for the management of MDR TB in the world, especially in low-resource countries where most cases of MDR TB occur. The turnaround time (TAT) is important in order for the patient to receive an appropriate treatment. The results obtained with the MGIT system, nitrate reductase assay and the colorimetric methods (MTT and resazurin) were available on average in 10 days as with the BACTEC 460 system. The time of detection for each method was almost the same. The INNO LiPA Rif.TB kit was used to confirm the resistance pattern of these strains but cannot be used in routine practice in low-resource countries due to its high cost. The BACTEC 460 system requires isotopes and heavy equipment, consequently is not feasible in most resource-poor settings. The commercial MGIT system, the non-radiometric alternative method, is reliable but still expensive to implement in low-resource countries. The three low cost methods: nitrate reductase assay, MTT and resazurin have been successfully tested in previous studies^{12–20} and might become inexpensive alternative methods for rapid and accurate detection of drug resistance of *M. tuberculosis* strains. Owing to their high level of agreement with the gold standard methods, they seem to have the potential to provide rapid detection of rifampicin resistance. These methods do not need any sophisticated equipment, are simple to perform, reduce the time to achieve results compared to the PM and could be implemented in laboratories with limited resources. One disadvantage of the MTT and resazurin assays is their biosafety since the plates use liquid medium and could generate aerosols. It has been shown recently that this format can be adapted to screw-cap tubes to avoid this situation.^{12,14,18} Nitrate reductase-negative strains of *M. tuberculosis* are very unusual²⁰ and on the other hand, false susceptible results would in this case be detected by the lack of a positive reaction also in drug-free growth. Current studies are in progress to evaluate these three low cost methods for all the first line drugs: rifampicin, isoniazid, streptomycin and ethambutol, with a large number of strains, to evaluate the reproducibility, specificity and sensitivity of these promising methods. It would also be interesting to assess the possibility of using these new techniques as direct assays.

Acknowledgements

We thank Professor Humberto Guerra for providing the strains from Peru. We also thank Pim De Rijk for his technical assistance. This study was supported by the European Commission RDG (INCO-DEV Programme), project number ICA4-CT-2001-10087 and by the Damian Foundation, Brussels, Belgium.

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