

Application of the resazurin microtitre assay for detection of multidrug resistance in *Mycobacterium tuberculosis* in Algiers

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This study assessed the performance of a rapid, low-cost, colorimetric method, the resazurin microtitre assay (REMA) plate method, for the detection of resistance to isoniazid and rifampicin in 136 clinical isolates of *Mycobacterium tuberculosis* from two hospitals in Algiers. MICs were determined and the results were compared with those obtained with the conventional proportion method on Löwenstein–Jensen medium. Excellent results were obtained for the REMA plate method, with a sensitivity of 100% for both isoniazid and rifampicin and a specificity of 98.3 and 99.2%, respectively. The REMA plate method appears to be a reliable method for the rapid determination of multidrug-resistant tuberculosis and is a good alternative for use in resource-limited countries such as Algeria.

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INTRODUCTION

Tuberculosis (TB) is a public health problem worldwide. According to the World Health Organization, 8.8 million new cases of TB were reported in 2003 and 1.7 million deaths were attributed to the disease (WHO, 2005). The human immunodeficiency virus pandemic and multidrug-resistant TB (MDRTB) have emerged as major obstacles in the treatment and efficient control of the disease (Barnes *et al.*, 2002; Corbett *et al.*, 2003; Frieden *et al.*, 2003). MDRTB is defined as TB that is resistant to at least rifampicin (RIF) and isoniazid (INH), the two most important drugs in the treatment of TB.

In Algeria, the incidence of TB in 2003 was notified as 53 cases per 100 000 inhabitants (WHO, 2005). For the year 2001, the prevalence of drug resistance was 6.2% and MDRTB in untreated patients amounted to 1.1% (WHO, 2004).

Effective treatment and prevention of MDRTB rely upon the prompt availability of drug-susceptibility testing (DST) results. For this reason, alternative, inexpensive and rapid methods of DST are needed urgently. The conventional agar and Löwenstein–Jensen (LJ) proportion method is laborious, with results only available after 3–6 weeks. The

BACTEC radiometric system (Becton Dickinson) had the advantage of being more rapid than the proportion method, but required the use of radioisotopes, which was a disadvantage with respect to the disposal of waste material and was expensive to perform (Siddiqi *et al.*, 1981; Roberts *et al.*, 1983). The Mycobacteria Growth Indicator Tube or MGIT (Palomino *et al.*, 1999; Goloubeva *et al.*, 2001) and the Etest (Wanger & Mills, 1996), both commercial methods, are simple and rapid to perform, but are still expensive, making them impractical for routine use in developing countries. Molecular methods for detection of drug resistance have also been described, such as the line probe assay INNO-LiPA (Innogenetics), but need substantial investment in equipment, which makes them impractical for routine use (De Beenhouwer *et al.*, 1995; Nachamkin *et al.*, 1997). In recent years, several new methods have been proposed for the rapid performance of DST of *Mycobacterium tuberculosis*, including phage assays (Simboli *et al.*, 2005) and cytofluorometry (Norden *et al.*, 1995; Moore *et al.*, 1999).

Recently, a new method using the oxidation–reduction colorimetric indicator resazurin has been proposed for the determination of drug resistance and MICs of antimicrobial agents against *M. tuberculosis* (Palomino *et al.*, 2002). Resazurin, which is blue in its oxidized state, turns pink when reduced by viable cells. The resazurin microtitre assay (REMA) plate method has been described for MIC determination with *M. tuberculosis* clinical isolates and has been tested successfully against INH and RIF for the detection of

Abbreviations: DST, drug-susceptibility testing; INH, isoniazid; MDRTB, multidrug-resistant tuberculosis; REMA, resazurin microtitre assay; RIF, rifampicin; TB, tuberculosis.

MDRTB (Gabrielson *et al.*, 2002; Palomino *et al.*, 2002, 2004).

This study describes the first application of the REMA plate method for DST of RIF and INH on clinical isolates of *M. tuberculosis* in two hospitals from Algeria. MIC results obtained with the REMA plate method were compared with DST performed by the conventional proportion method on LJ medium.

METHODS

Mycobacterial isolates. The study was performed on 136 *M. tuberculosis* clinical isolates originating from 136 patients. The isolates were identified as *M. tuberculosis* by conventional culture and biochemical tests (Kent & Kubica, 1985). One hundred and sixteen isolates were collected between June 2000 and December 2004 at the mycobacteriology laboratory of El Hadi Flici Hospital and 20 isolates were collected between June 2000 and June 2001 at the Service of Pneumophtisiologie, Matiben, of the Issad Hacene Hospital, both in Algiers.

Antibiotics. INH stock solution (1 mg ml⁻¹) was prepared in distilled water and RIF stock solution (10 mg ml⁻¹) was prepared in methanol. Both solutions were sterilized by filtration through a 0.2 µm membrane and stored at -20 °C until use.

Resazurin reagent. The resazurin reagent was obtained as resazurin sodium salt powder (Acros Organic NV). A working solution was prepared at a concentration of 0.01% (w/v) in distilled water and sterilized by filtration through a 0.2 µm membrane; this working solution was stored at 4 °C for up to 1 week.

Culture medium. For the REMA plate method, 7H9-S medium was used consisting of Middlebrook 7H9 broth containing 0.1% casitone, 0.5% glycerol and 10% oleic acid, albumin, glucose and catalase supplement (Becton Dickinson).

REMA plate method. The REMA plate method was performed as described by Palomino *et al.* (2002). Briefly, the INH and RIF stock solutions were diluted in 7H9-S to four times the final highest concentration tested. Serial twofold dilutions of these solutions were prepared in a 96-well microtitre plate using 100 µl 7H9-S. The range of concentrations tested was 1.00–0.03 µg ml⁻¹ for INH and 2.00–0.06 µg ml⁻¹ for RIF. A growth control containing no antibiotic and a sterility control without inoculum were included in each plate. The inoculum was prepared by resuspending a loopful of the LJ culture medium in a tube containing 3 ml 7H9-S medium with several

glass beads. The tube was vortexed for 2 min and sediment was allowed to form for 15 min. The supernatant was transferred to a second tube and the turbidity adjusted to match a McFarland tube no. 1 standard; this suspension was further diluted 1:20 in 7H9-S. The plates were inoculated with 100 µl suspension and sealed in plastic bags; incubation was at 37 °C in a normal atmosphere. After incubation for 7 days, 30 µl resazurin working solution was added to each well; the plates were incubated for 24 h at 37 °C and the results were read visually. A change in colour of the resazurin from blue to pink indicated reduction of the indicator and thus bacterial growth. For a positive result, the colour change indicating growth had to be comparable to that observed in the positive growth control. The MIC was defined as the lowest drug concentration that prevented a full colour change of the resazurin from blue to pink. According to Palomino *et al.* (2002), the criterion for resistance or susceptibility is defined as follows: for INH, a strain is considered resistant if the MIC is ≥0.25 µg ml⁻¹; for RIF, a strain is considered resistant if the MIC is ≥0.5 µg ml⁻¹.

Proportion method. The proportion method was performed according to established procedures on LJ medium with critical concentrations of 0.2 µg ml⁻¹ for INH and 40 µg ml⁻¹ for RIF (Canetti *et al.*, 1963, 1969). A strain was classified as susceptible to the drug if the number of colonies that grew on the drug-containing medium was <1% of the number of colonies that grew on the control tube and resistant if the number of colonies was >1%.

RESULTS AND DISCUSSION

This study involved 136 *M. tuberculosis* isolates, each from a different patient. Results of the REMA plate method were obtained after 8 days of incubation. For INH, of the 119 isolates found to be susceptible according to the proportion method, 117 had an MIC of 0.125 µg ml⁻¹ or lower; one isolate had an intermediate MIC of 0.25 µg ml⁻¹ and one isolate gave a discordant result, being susceptible by the proportion method but resistant by the REMA plate method, with an MIC of >1 µg ml⁻¹. This isolate was retested by both methods and the same result was obtained. The 17 isolates found to be resistant according to the proportion method had MICs of >1 µg ml⁻¹ (Table 1). For RIF, of the 124 isolates found to be susceptible by the proportion method, 123 had an MIC of ≤0.25 µg ml⁻¹. One isolate that was found to be susceptible by the proportion method showed an intermediate resistance in the REMA plate method with an MIC of 0.5 µg ml⁻¹. Of

Table 1. MICs of INH and RIF for 136 *M. tuberculosis* isolates determined by the REMA plate method

Proportion method	No. of isolates for which MIC (µg ml ⁻¹) of INH was:						
	≤0.03	0.06	0.125	0.25	0.1	1.0	>1.0
Resistant (n=17)	0	0	0	0	0	0	17
Susceptible (n=119)	94	12	11	1	0	0	1
	No. of isolates for which MIC (µg ml ⁻¹) of RIF was:						
	≤0.06	0.125	0.25	0.5	1.0	2.0	>2.0
Resistant (n=12)	0	0	0	1	1	0	10
Susceptible (n=124)	88	24	11	1	0	0	0

the 12 isolates found to be resistant by the proportion method, 11 were also resistant according to the results of the REMA plate method, with MIC values of $\geq 1 \mu\text{g ml}^{-1}$, whilst the remaining isolate had an intermediate MIC of $0.5 \mu\text{g ml}^{-1}$ (Table 1). According to these results, the sensitivity of the REMA plate for INH and RIF was 100% and the specificity was 98.3 and 99.2%, respectively.

Several studies have stressed the importance of timely detection of drug resistance in TB (Seung *et al.*, 2004). Delays in detecting drug resistance have proved to be fatal in human immunodeficiency virus-infected patients with MDRTB. DST of new isolates from new patients is an essential component of the DOTS (directly observed treatment, short course) strategy (Iseman, 1998), but it takes between 3 and 6 weeks to obtain results by the conventional proportion method. This study evaluated the recently described REMA plate method for rapid DST of *M. tuberculosis* isolates in Algeria with good results. Results were obtained in a short period of time and with a very good sensitivity and specificity compared with the proportion method, which took several weeks to confirm the results. Our results confirm recent reports of the performance of the REMA plate method in other settings (Martin *et al.*, 2003; Lemus *et al.*, 2004; Montoro *et al.*, 2005). The cost of the test will vary according to the setting and where it is used; however, it compares favourably with the conventional proportion method in LJ medium. One important concern of this type of test performed in microtitre plates with liquid medium relates to the biosafety requirements. This can be overcome by performing the test in individual closed tubes; however, the test is recommended for reference laboratories that already have the necessary biosafety facilities.

Early detection of drug resistance and MDRTB is very important for adequate control of TB and for starting the appropriate treatment for the patient. The REMA plate method appears to be a good alternative method for use in low-resource countries such as Algeria.

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