



Original Research Article

Evaluation of Drug Susceptibility to Rifampicin, INH and Linezolid using Resazurine Microtitre Assay

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ABSTRACT

In the present study we have evaluated rapid low cost Resazurin microtitre assay (REMA) for drug susceptibility for rifampicin, INH and Linezolid on 90 clinical isolates of *M. tuberculosis* (29 MDR, 56 non-MDR and 5 XDR). MIC results obtained with the REMA plate method were compared with DST performed by the conventional proportion method on LJ. The INH MICS for all 45 *M. tuberculosis* isolates resistant to INH by proportion method were ≥ 0.25 $\mu\text{g}/\text{ml}$ by REMA. Rifampicin MIC for 34 isolates shown to be resistant to rifampicin by proportion method had MIC of ≥ 0.5 $\mu\text{g}/\text{ml}$ by REMA. For linezolid MIC for all 90 isolates ranged from 0.125 to 1 $\mu\text{g}/\text{ml}$ by REMA but 30/90 isolates showed growth on LJ containing 1.2 $\mu\text{g}/\text{ml}$ of linezolid. REMA method had sensitivity of 100% for INH susceptibility and specificity of 97.7 while for rifampicin susceptibility sensitivity and specificity both was 100%. All 90 isolates were detected as sensitive to linezolid ($\text{MIC} \leq 1$ $\mu\text{g}/\text{ml}$) by REMA but 30/90 showed growth on LJ containing 1.2 $\mu\text{g}/\text{ml}$ linezolid. Thus proportion method needs to have change in that critical concentration for linezolid. The present observations suggest that results of REMA are available in 7 days against 4–6 weeks for proportion method on LJ and is a good candidate for rapid detection of susceptibility for anti TB drugs and is economical as well. The most important finding was that all 90 isolates including MDR and XDR were found to be susceptible to linezolid *in-vitro*.

Keywords

Mycobacteria,
REMA,
Linezolid,
Tuberculosis,
MIC

Introduction

Tuberculosis (TB) is still a major public-health problem all over the world, particularly in developing countries. According to the latest World Health Organization (WHO) report in 2014, there were 9 million new TB cases and 1.5 million deaths attributed to the disease worldwide (Global Tuberculosis Control 2014). The situation becomes more complicated due to

the rising human immunodeficiency virus/AIDS pandemic, the emergence of multidrug-resistant (MDR) TB and the recently described extensively drug-resistant TB (Aziz *et al.*, 2006; Shah *et al.*, 2007).

Therefore, new anti-TB drugs are urgently needed to treat MDR and XDR-TB (Extensively drug resistant tuberculosis is

defined as resistance to at least Isoniazid and Rifampicin (i.e. MDR-TB) plus resistance to any of the fluoroquinolones and any one of the second-line injectable drugs (amikacin, kanamycin, or capreomycin). Linezolid was the first oxazolidinone compound licensed for clinical use. It has been suggested as an alternative treatment for patients infected with MDR *M. tuberculosis* isolates (Dietze *et al.*, 2008; Cynamon *et al.*, 1999). However, there are only few *in-vitro* studies of linezolid's activity against clinical *M. tuberculosis* isolates (Wallace *et al.*, 2001; Rodriguez *et al.*, 2002, 2004; Barbara *et al.*, 2003; Luis Alcala *et al.*, 2003; Zayre Erturan *et al.*, 2005; Tato *et al.*, 2006; Richter *et al.*, 2007; Prammananan *et al.*, 2009; Ermertcan *et al.*, 2009; Caie Yang *et al.*, 2012).

Effective treatment and prevention of MDR-TB rely upon the prompt availability of drug-susceptibility testing (DST) results. For this reason, alternative, inexpensive and rapid methods of DST are needed urgently. Current standard methods for the detection of MDR *Mycobacterium tuberculosis* include the proportion method (PM) performed on Löwenstein–Jensen (LJ) medium or agar, absolute concentration and resistance ratio methods (Canetti *et al.*, 1963, 1969; Kent and Kubica, 1985) and the radiometric method in the BACTEC-460 system (Siddiqi *et al.*, 1981; Roberts *et al.*, 1983). However, results of these methods either take longer time or produce radioactive waste that is difficult to manage in low-resource countries. The Mycobacteria Growth Indicator Tube or MGIT (Palomino *et al.*, 1999; Goloubeva *et al.*, 2001) and the E test (Wanger and Mills, 1996) both commercial methods, are simple and rapid to perform, but are 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 (Mogahid *et al.*, 2014) 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 MDR-TB (Palomino *et al.*, 2002, 2004; Martin *et al.*, 2005, 2011; Nateche *et al.*, 2006; Rivoire *et al.*, 2007; Affolabi *et al.*, 2008; Ahmet *et al.*, 2012).

In the present study we have evaluated rapid low cost REMA plate method for DST of RIF, INH and Linezolid on clinical isolates of *M. tuberculosis*. MIC results obtained with the REMA plate method were compared with DST performed by the conventional proportion method on LJ.

Materials and Methods

Mycobacterial isolates

The study was performed on 90 clinical isolates (29 MDR, 56 non-MDR and 5 XDR) of *M. tuberculosis* originating from 136 patients. The isolates were identified as

M. tuberculosis by conventional culture and biochemical tests (Kent and Kubica, 1985). The susceptibility to INH and Rifampicin was as below.

INH resistant	INH susceptible	RIF resistant	RIF susceptible
45	45	43	56

Antibiotics

Stock concentration of INH drug: 10 mg /10 ml drug solution (Sigma-Aldrich Chemicals GmbH, Steinheim, Germany) was prepared in distilled water and after passing through membrane filter; aliquots were made and kept at -70°C.

Stock concentration of RIF: Rif (Sigma-Aldrich Chemicals GmbH, Steinheim, Germany) was made up as stock solution at 10 mg /10 ml in Dimethylformamide (BDH, England) and stored at -70°C in 0.5 ml vials until use. Further dilutions were made in Middlebrook 7H9 broth (Difco, USA).

Stock concentration of linezolid drug: 20 mg /10 ml drug solution (Cadila, India) was prepared in distilled water and after passing through membrane filter; aliquots were made and kept at -70°C.

Resazurin reagent

The resazurin reagent was obtained as resazurin sodium salt powder (Hi-media, India). 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, USA).

Preparation of bacterial inocula

Approximately five-six colonies that had been freshly grown on Löwenstein-Jensen media were inoculated in to physiologic saline in tubes containing 5-10 glass beads and then vortexed for 1-2 min. The tube was kept in a vertical position for 30 min at room temperature to allow for the sedimentation of aerosols and large particles. The turbidity of the supernatant was adjusted to that of the McFarland tube no. 1 standard. The adjusted bacterial suspension was then diluted (1:10 ratio). One hundred microlitres of the diluted mycobacterial suspension was used for the inoculation (Martin *et al.*, 2011).

REMA plate method

The REMA plate method was performed as described by Palomino *et al.* (2002). Briefly, the INH, RIF and linezolid stock solutions were diluted in 7H9-S medium to four times the final highest concentration tested. Serial twofold dilutions of these solutions were prepared in a 96-well microtitre plate using 100µl 7H9-S medium. The range of concentrations tested was 1.00–0.03 µg/ml for INH, 2.00–0.06 µg/ml for RIF and 1.00–0.125 µg/ml for linezolid. A growth control containing no antibiotic and a growth control without inoculum were included in each plate. The plates were inoculated with 100µl suspension and sealed in plastic bags; incubation was at 37°C in a humid atmosphere.

After incubation for 7 days, 37 μ l 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 μ g/ml, for RIF a strain is considered resistant if the MIC is ≥ 0.5 μ g/ml and for linezolid a strain is considered resistant if the MIC is ≥ 8 μ g/ml (Wallace *et al.*, 2001).

Proportion method

The proportion method was performed according to established procedures on LJ medium with critical concentrations of 0.2 μ g/ml for INH and 40 μ g/ml for RIF (Canetti *et al.*, 1963, 1969) and 1.2 μ g/ml for Linezolid (Rodriguez *et al.*, 2004). 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 90 *M. tuberculosis* isolates, each from a different patient. Result of the REMA plate method was observed after 8 days of incubation. Results obtained with REMA and the proportion method on LJ medium are compared in the table 1. The

INH MICs for all 45 *M. tuberculosis* isolates determined to be resistant to INH by PM were at least 0.25 μ g/ml with the REMA plate method, and the MICs were 1.0 μ g/ml or higher for 36 (80%) of the isolates. MICs of 0.06 μ g/ml or lower were observed by using the REMA plate method for 44 out of 45 isolates determined to be susceptible to INH by PM (Table 1).

The only discordant isolate was determined to be susceptible by PM, but the MIC for it was higher than 1 μ g/ml with the REMA plate method. On repeated testing, it was found to give the same result by both methods. Based on these results, the tentative breakpoint concentration of INH was defined as 0.25 μ g/ml.

RIF MICs for all 34 isolates shown to be resistant to RIF by PM were at least 0.5 μ g/ml with the REMA plate method, and the MICs for all but one (33 out of 34) were higher than 2 μ g/ml. MICs for all 56 isolates shown to be susceptible by PM were 0.25 μ g/ml or lower when tested by the REMA plate method (Table 1). A tentative breakpoint concentration of RIF was defined as 0.5 μ g/ml.

For INH, 79 out of 80 results were concordant, yielding specificity and sensitivity of 97.7% and 100%, respectively, with positive predictive values and negative predictive values 97.8% and 100%. As all 90 results were concordant for RIF, the specificity, sensitivity, and predictive values were all 100%.

The susceptibility to linezolid by REMA plate method for all 90 (29 MDR, 56 non-MDR and 5 XDR) of the *M. tuberculosis* isolates examined is shown in table 2. The MICs for linezolid ranged from 0.125 to 1 μ g/ml for all tested isolates. The MIC against *M. tuberculosis* H37Rv was 1 μ g/ml. All isolates were inhibited between 0.125 to

1 µg/ml and by REMA plate method (Table 2). However, 30 of the 90 isolates showed growth on LJ slants containing 1.2 µg/ml linezolid. The spread of MDR strain could seriously jeopardize the fight against TB. The diagnosis of MDR TB in India is not easy because the only available method to determine drug resistance is standard proportion method on LJ medium, which requires about 4-6 weeks to yield result. Liquid culture method such as the BACTEC TB 640 system and molecular method such as INNO LiPA are very much quicker, but these technologies cannot be used in low income countries because they are expensive and require special equipment. Thus rapid simple test for the identification of MDR would be useful, for earlier detection of MDR strains which will help in the management of and prevent their transmission.

A comparison of the quantitative results obtained by the proportion method on LJ medium and MIC result for INH and RIF by REMA led to conclusion that the breakpoint concentration determined by Palomino *et al.* (2004) are appropriate. Using these breakpoints, detection of resistance to INH and RIF by REMA showed good sensitivity (100% for both drug) and specificity (97.7% and 100%) with references to the gold standard method, with INH and RIF cut off values of respectively 0.25µg/ml and 0.5µg/ml. Our results are similar to the study found specificity and sensitivity of 96.2% and 100%, respectively, with predictive values for susceptibility and resistance of 100% and 98.2% for INH, and for RIF the specificity, sensitivity, and predictive values were all 100%. Mortine *et al.* (2011) found very good sensitivity 99.1% specificity 100% for INH, and sensitivity, specificity 100% for RIF.

Several studies have previously measured the *in-vitro* activity of linezolid against *M.*

tuberculosis isolates. Richter *et al.* (2007) evaluated the activity of linezolid against 210 MDR-TB isolates and found the first isolates that could not be inhibited by concentration of linezolid < 8 µg/ml. Prammananan *et al.* (2009) investigated a large number of MDR-TB isolates, including 9 XDR-TB isolates, in Thailand and found that 2 isolates that were not inhibited by a concentration of linezolid < 6 µg/ml. Ermertcan *et al.* (2009) reported good activity of linezolid against 67 isolates of *M. tuberculosis* (33 MDR and 34 non-MDR isolates) in western Turkey, with MICs of 0.06–1 µg/ml. Caie Yang *et al.*, (2012) reported good activity of linezolid against 84 isolates of *M. tuberculosis* in China with MICs of 0.125–0.5µg/ml while reported MIC for 61 isolates (including 20 MDR and 10 XDR) in the range of 0.125-1 µg/ml. These data suggest that activity of linezolid against TB varies across different geographic areas.

In the present study, linezolid showed excellent *in-vitro* activity against all *M. tuberculosis* isolates tested and inhibited both MDR and XDR isolates with MICs of 1.0–0.125 µg/ml. Further, it appears that MIC on LJ will be higher than by liquid culture (REMA plate method) and the proportion method needs to have higher-break point for interpretation as sensitive and resistant.

Used as indirect test, REMA was rapid (8 days after culture on solid medium vs.3–6 weeks for the indirect testing on LJ medium) and technically simple. The total turnaround time was 4–5 weeks for REMA vs.7–10 weeks for LJ. It was also economical (including the cost of reagent and consumables) than the proportion method using LJ medium. The REMA method could therefore prove useful for both the diagnosis of MDR and primary resistance surveys in low income countries.

Table.1 Comparing the result of REMA with Proportion method

Comparing the result of REMA with Proportion method							
Resazurin microtiter assay for Rif and INH							
Drug	Proportion method	resistant	susceptible	sensitivity	specificity	PPV	NPV
INH	Resistant (<i>n</i> =45)	45	0	100%	97.7%	97.8%	100%
	Susceptible (<i>n</i> =45)	1	44				
RIF	Resistant (<i>n</i> =34)	34	0	100%	100%	100%	100%
	Susceptible (<i>n</i> =56)	0	56				

Table.2 Activity of linezolid against 90 *M. tuberculosis* isolates (29 MDR, 56 non MDR and 5 XDR)

On the basis of Proportion Method using LJ	No. of isolates for Linezolid MIC (ug/ml) by REMA			
	0.125	0.25	0.5	1
MDR (R to RIF & INH) (<i>n</i> = 29)	0	0	19	10
XDR (<i>n</i> = 5)	0	0	2	3
Non MDR (S to RIF & INH) (<i>n</i> = 56)	0	11	25	20
H37Rv	0	0	0	1

One disadvantage of the REMA test is risk of contamination, as it is carried out in microplates using liquid medium.

In summary, the REMA test was found to be reliable, inexpensive and simple to perform. In addition, it required less cumbersome incubators than those needed for the proportion method involving LJ medium. Furthermore, unlike the molecular methods, it does not require sophisticated equipment. The minimum major equipment requirement for performing this test included a level P2 biosafety cabinet, an aerosol contained centrifuge and a 37°C incubator. We conclude that the REMA test could easily be implemented and might even replace the proportion method in central laboratories in low-resource countries such as India. There is an urgent need to evaluate REMA for

determining resistance to other first and second line TB drugs.

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