Resazurin Microtiter Assay Plate method for detection of susceptibility of multidrug resistant *Mycobacterium tuberculosis* to second-line anti-tuberculous drugs

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**KEYWORDS**

*Mycobacterium tuberculosis* (M. tuberculosis); Minimal inhibitory concentration (MIC) evaluation; Resazurin; Second-line anti tuberculous drugs

**Abstract**

**Background:** Many developed countries need a diagnostic test that is accurate, rapid, and economical in detecting multidrug-resistant tuberculosis. This study aims to evaluate Resazurin Microtiter Assay Plate in detecting susceptibilities of multidrug resistant *Mycobacterium tuberculosis* to second line anti tuberculous drugs.

**Methods:** Susceptibility of multidrug resistant *M. tuberculosis* was evaluated against 5 second line anti tuberculous drugs by the colorimetric method using the Resazurin Microtiter Assay Plate Testing (REMA).

**Results:** Drug susceptibility testing for the second-line drugs was performed for 34 MDR *M. tuberculosis* isolates isolated from 40 sputum samples. Results of REMA were available after 8 days of incubation. The agreement between the two tests for para-aminosalicylic acid, ethionamide, ofloxacin, kanamycin monosulfate and cycloserine was found to be 97.05%, 94.11%, 97.05%, 97.05% and 100%, respectively. The efficacy of REMA plate assay for PAS, ETH, OFX, KAN and CYL was 97.05%, 94.11%, 97.05%, 97.05% and 100%, respectively.

**Conclusion:** Resazurin test was found to be reliable, simple to perform for the rapid detection of anti-tuberculous drug resistance and economically inexpensive.

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**Introduction**

Tuberculosis remains an important public health problem and has recently been increasing in incidence [1]. Tuberculosis (TB) is a serious public health problem in many developing countries. According to the World Health Organization (WHO) report in 2005, there were 8.8 million new TB Cases and 1.6 million deaths were attributed to the disease worldwide [2].
Egypt is ranked as a country with middle/low level of tuberculosis (TB) incidence. It is estimated that 11 cases per 100,000 population develop active pulmonary smear positive TB annually, while 24 per 100,000 develop all types of TB annually according to the World Health Organization (WHO) report in 2010 [3]. Multidrug resistant (MDR) Mycobacterium tuberculosis strains have been observed around the world, with high mortality rates particularly among HIV-positive patients [4–6]. MDR TB is now also emerging in HIV-negative populations and in high-risk populations such as medical staff [7,8]. MDR M. tuberculosis isolates present a major challenge for tuberculosis control programs [9]. Although progress has been made to reduce global incidence of drug-susceptible tuberculosis, the emergence of MDR and extensively drug-resistant (XDR) tuberculosis during the past decade threatens to undermine these advances. However, countries are responding far too slowly. Of the estimated 440,000 cases of MDR tuberculosis that occurred in 2008, only 7% were identified and reported to WHO. Of these cases, only a fifth were treated according to WHO standards [10]. MDR-TB patients do not respond to treatment with the first-line drugs isoniazid (INH), rifampin (RIF), ethambutol (EMB), pyrazinamide (Z) and streptomycin (SM) [11–14]. Consequently, the treatment of MDR-TB involves the use of reserve drugs called ‘second-line drugs’ [15]. Several studies have shown that MDR TB can be cured by a combination of second-line drugs under DOTs-Plus, the treatment strategy proposed by the World Health Organization to address the management of MDR TB in settings with good control programs [14–19]. However, these drugs are expensive, have to be taken for long periods, and can cause adverse reactions [14]. Rapid diagnosis of MDR patients is nevertheless necessary to avoid the spread of MDR strains. Therefore, microbial susceptibility should be ascertained to diagnose MDR tuberculosis. The test should be specific, inexpensive, rapid, simple and applicable to developing countries [20]. 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 materials and was expensive to perform [21,22]. In recent years, several new methods have been proposed for the rapid performance of Drug susceptibility testing (DST) of M. tuberculosis, including phage assays [23] and cytofluorometry [24,25].

The development of new, rapid Drug susceptibility testing which is easy to use and inexpensive is thus an urgent priority for determining the susceptibility to second line drugs [26,27]. A new method using the oxidation–reduction colorimetric indicator resazurin has been proposed for the determination of drug resistance and minimal inhibitory concentration (MICs) of antimicrobial agents against M. tuberculosis [28–31]. Resazurin, which is blue in its oxidized state, turns pink when reduced by viable cells. The REMA plate method has been described for MIC determination of M. tuberculosis clinical isolates and has been tested successfully against INH and RIF for the detection of MDRTB [28,32,33].

Aim of this work

The study evaluated Resazurin Microtiter Assay Plate in the detection of susceptibilities of MDR M. tuberculosis to second-line anti-tuberculous drugs. The results were compared to those obtained by the standard proportional method (PM) performed on Löwenstein–Jensen medium (LJ).

Subjects and methods

Patients

Forty patients (28 males and 12 females) suffering from multi-drug resistant pulmonary tuberculosis (MDR) with age ranging from 20 to 62 years, who attended MDR ward at Abbassia chest hospital in the period from December 2011 to April 2012 were chosen for the study.

Methods

Susceptibility of multidrug resistant M. tuberculosis was evaluated against 5 second-line anti-tuberculous drugs: ethionamide (ETH), kanamycin monosulfate (KAN), cycloserine (CYL), ofloxacin (OFX), and para-aminosalicylic acid (PAS) by the colorimetric method using the REMA Plate Testing and the results were compared to those obtained using the proportional method (PM), considered as the gold standard. All sputum specimens were digested and decontaminated of other bacteria by the standard N-acetyl-L-cysteine–NaOH–sodium citrate method followed by centrifugation and concentration according to Kubic et al. [34], then cultured on LJ medium. Six samples yielded negative cultures and 34 isolates were identified by niacin accumulation test (Becton Dickinson, USA), growth inhibition by thiophene-2-carboxylic acid hydrazide (Sigma–Aldrich, USA) and pyrazinamidase test (Himedia, India). ETH, OFX, CYL, KAN and PAS were obtained from Sigma Aldrich, India. Stock solutions at 1 mg/ml were filter sterilized and stored at -20 °C. Working solutions were prepared at four times the final higher concentration in 7H9-S broth (Middlebrook 7H9 supplemented with 0.1% Casitone, 0.5% glycerol, and 10% OADC [oleic acid, albumin, dextrose, and catalase]; Becton–Dickinson). The final drug concentrations tested were as follows: for PAS and OFX, 8 µg/ml; for ETH and KAN and CYL, 20 µg/ml. A stock solution of resazurin sodium salt powder (Sigma Aldrich, China) was prepared at 0.02% (wt/vol) in distilled water, sterilized by filtration, and stored at 4 °C for up to 1 week. The inoculum was prepared from fresh LJ medium in 7H9-S broth, adjusted spectrophotometrically to a no. 1 McFarland tube standard, and further diluted 1:10 in 7H9-S broth for the test. The REMA plate assay (Fig. 1) was performed according to Taneja and Tyagi [35] and Martin et al. [36]. Briefly, 100 µl of 7H9-S broth was dispensed in each well of a sterile flat-bottom 96-well plate (Becton Dickinson, USA) and serial two fold dilutions of each drug were prepared directly in the plate. One hundred microliters of inoculum was added to each well (2 isolates per plate). A growth control and a sterile control were also included for each isolate. 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 under 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 from blue to pink indicates reduction of resazurin and therefore bacterial growth. A change in color from blue to pink indicated the growth of bacteria, and the minimal
inhibitory concentration (MIC) was defined as the lowest concentration of the drug that prevented this change in color. A change in color of growth control well to pink indicated the proper growth of the isolate and no change in color of sterile control well indicated absence of contaminants. The drug concentration ranges used were as follows: 20–0.62 \( \mu \text{g/ml} \) for ETH, KAN and CYL and 8–0.25 \( \mu \text{g/ml} \) for OFX and PAS.

**(Drug cut-off points by REMA)**: 2.5 \( \mu \text{g/ml} \) for ETH, 5 \( \mu \text{g/ml} \) for KAN and CYL and 2 \( \mu \text{g/ml} \) for OFX and PAS [37]. PM was performed on the LJ medium according to laboratory’s standard procedure [38] using critical concentration of drugs mentioned above.

**Statistical methods**

IBM SPSS statistics (V. 21.0, IBM Corp., USA, 2012) was used for data analysis. Data were expressed as both number and percentage for categorized data. Diagnostic validity test was performed and included: the diagnostic sensitivity (the percentage of resistant cases truly diagnosed (TP) among total resistant cases (TP + FN)), the diagnostic specificity (the percentage of susceptible cases truly excluded by the test (TN) among total susceptible cases (TN + FP)) and the efficacy or the diagnostic accuracy of the test (the percentage of resistant cases truly diagnosed plus susceptible cases truly excluded by the test among total cases).

TP (true positive), FN (false negative), FP (false positive), TN (true negative).

**Results**

DST for the second-line drugs OFX, KAN, CYL, ETH, and PAS was performed for 34 MDR *M. tuberculosis* isolates isolated from 40 sputum samples. Results of REMA were available after 8 days of incubation, while those of testing by the PM were available after 3–4 weeks. The DST results for the second-line drugs obtained by PM were as follows: 4 isolate (11.7%) were resistant to PAS, 14 (41.1%) were resistant to ETH, 6 (17.6%) were resistant to OFX, 4 (11.7%) were resistant to KAN, and 2 (5.9%) were resistant to CYL. As regards REMA plate method; number and MICs for five second-line drugs of resistant isolates are shown in Table 1.

**Table 1** shows that MIC detected by REMA among PAS resistant isolates (no. = 3) was 4 \( \mu \text{g/ml} \) (2 isolates) and 8 \( \mu \text{g/ml} \) (1 isolate). Among ETH resistant isolates (no. = 12) MIC was 5 \( \mu \text{g/ml} \) (7 isolates), 10 \( \mu \text{g/ml} \) (3 isolates) and 20 \( \mu \text{g/ml} \) (2 isolates). Among OFX resistant isolates (no. = 5), detected MIC was 4 \( \mu \text{g/ml} \) (3 isolates) and 8 \( \mu \text{g/ml} \) (2 isolates). For KAN, MIC of resistant isolates (no. = 5) was 5 \( \mu \text{g/ml} \) (4 isolates) and 10 \( \mu \text{g/ml} \) (1 isolate). For CYL, MIC of resistant isolates (no. = 2) was 5 \( \mu \text{g/ml} \) (1 isolate) and 20 \( \mu \text{g/ml} \) (1 isolate).

**Table 2** and **Fig. 2** show the DST results obtained by the REMA plate method compared with the PM, considered the gold standard. For PAS, both methods detected 30 susceptible isolates and 3 resistant isolates, 1 isolate gave a discrepant result as resistant by PM while being susceptible by REMA. For ETH, 20 isolates were detected as susceptible and 12 as

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**Table 1** MICs of five second-line drugs for resistant isolates detected by REMA plate method:

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (( \mu \text{g/ml} ))</th>
<th>Number of isolates</th>
</tr>
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<tbody>
<tr>
<td>PAS</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
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<td>3</td>
</tr>
<tr>
<td>ETH</td>
<td>5</td>
<td>7</td>
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<tr>
<td></td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>12</td>
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<tr>
<td>OFX</td>
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<tr>
<td></td>
<td>8</td>
<td>2</td>
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<tr>
<td>Total</td>
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<td>5</td>
</tr>
<tr>
<td>KAN</td>
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<td>4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>CYL</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1</td>
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<tr>
<td>Total</td>
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<td>2</td>
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</table>
resistant by both methods; 2 isolates gave discrepant results as resistant by PM and susceptible by REMA. For OFX, 5 and 28 isolates were identified as resistant and susceptible respectively by both methods while 1 isolate was misidentified as susceptible by REMA plate assay. For KAN, 4 isolates were correctly detected as resistant and 29 as sensitive by both methods; 1 isolate was false-positive by REMA plate assay. For CYL, 32 isolates were detected as susceptible and 2 isolates as resistant by both methods and no discrepant results were detected.

The agreement between the two tests for PAS, ETH, OFX, KAN and CYL was 97.05%, 94.11%, 97.05%, 97.05% and 100%, respectively.

### Discussion

The emergence of drug-resistant *M. tuberculosis* is a global concern, the early detection of antimicrobial susceptibility is especially important for creating an appropriate treatment protocol for patients with TB [9]. Several studies have stressed the importance of timely detection of drug resistance in TB [39]. Laboratories supporting TB services in areas with a high prevalence of MDR TB must therefore be able to provide prompt and reliable DST for drugs used in the management of patients [40]. Preliminary studies of DST of MDR TB isolates using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Alamar blue, or resazurin showed a good correlation with the conventional PM [28,41–45]. The limitation of some proprietary dyes is the lack of knowledge of their compositions [46]. Recently, resazurin, a nonproprietary product, has been identified as the main component of Alamar blue [47,48]. The REMA plate method has proven to be, in recent experience, a reliable method for the detection of MDR TB [28].

The current study was carried upon 40 patients suffering from MDR TB, DST of 34 *M. tuberculosis* isolates has been performed with five second-line drugs by the REMA plate method. Results of the REMA plate method were obtained after 8 days of incubation, while those of testing by the PM were available after 3–4 weeks. The DST results for the second-line drugs obtained by PM were as follows: 4 isolate (11.7%) were resistant to PAS, 14 (41.1%) were resistant to ETH, 6 (17.6%) were resistant to OFX, 4 (11.7%) were resistant to KAN, and 2 (5.9%) were resistant to CYL. The REMA plate method has also shown a high level of agreement with the conventional PM.
Results were easily determined visually by reading the change to a stable color from blue to pink. The agreement between the two tests for PAS, ETH, OFX, KAN and CYL was found to be 97.05%, 94.11%, 97.05%, 97.05% and 100%, respectively.

These results confirm recent reports of the performance of the REMA plate method in other settings. Martin et al. [36] examined susceptibilities to second-line drugs by using the REMA Plate Testing and found among the 150 isolates, 31 were resistant to ETH, 29 were resistant to KAN, 9 were resistant to PAS, 8 were resistant to OFX, and 19 were resistant to CAP by the PM. Complete agreement in results was found for KAN, PAS, and OFX by the two methods.

The current study showed the sensitivity of the REMA plate assay compared to that of PM was observed to be 75%, 85.71%, 83.33% for PAS, ETH and OFX respectively and 100% for both KAN and CYL. Specificities were 100% for PAS, ETH, OFX and CYL and 96.67% for KAN. The efficacy of REMA plate assay for PAS, ETH, OFX, KAN and CYL was 97.05%, 94.11%, 97.05%, 97.05% and 100%, respectively.

Other studies have reported a higher sensitivity. Martin et al. [36] found that, among the 150 isolates, the overall performance of the REMA plate method for the five antimicrobial agents in comparison with that of the PM was very good. The specificity was 100% for the five drugs ethionamide, kanamycin, capreomycin, ofloxacin, and para-aminosalicylic acid, and the sensitivity ranged from 96.8% to 100%; for CAP the sensitivity was 84.2%. The observed discrepancy between these results might be attributed to the limited number of patients enrolled in the present study and different geographical distributions. Also these studies included different resistant isolates than the present study and the precision of the sensitivity estimates was accordingly lower in this study.

According to the current study REMA was a rapid and an inexpensive method with all results available in 8 days. REMA has also the advantage of easily determining the MIC; MIC detected by REMA among PAS resistant isolates (no. = 3) was 4 μg/ml (2 isolates) and 8 μg/ml (1 isolate). Among ETH resistant isolates (no. = 12). MIC was 5 μg/ml (7 isolates), 10 μg/ml (3 isolates) and 20 μg/ml (2 isolates). Among OFX resistant isolates (no. = 5), detected MIC was 4 μg/ml (3 isolates) and 8 μg/ml (2 isolates). For KAN, MIC of resistant isolates (no. = 5) was 5 μg/ml (4 isolates) and 10 μg/ml (1 isolate). For CYL, MIC of resistant isolates (no. = 2) was 5 μg/ml (1 isolate) and 20 μg/ml (1 isolate).

These results were in accordance with Martin et al. [36] who reported that the MIC by REMA among PAS resistant isolates (no. = 9) was >8 μg/ml (9 isolate). Among ETH resistant isolates (no. = 31) was 1.25 μg/ml (1 isolate), 5.0 μg/ml (4 isolates), 10.0 μg/ml (7 isolates) and >20 μg/ml (19 isolates). Among OFX resistant isolates (no. = 8), detected MIC was 4 μg/ml (4 isolates) and >8 μg/ml (4 isolates). For KAN, MIC of resistant isolates (no. = 29) was 5 μg/ml (4 isolates), 10 μg/ml (3 isolate) and >20 μg/ml (22 isolates).

The MICs of ofloxacin resistant *M. tuberculosis* isolates (no. = 8) were determined by Martin et al. [40]. MIC detected by REMA was 4 μg/ml (5 isolates) and 8 μg/ml (3 isolates). At 2 μg/ml of OFX, the specificity and sensitivity were both 100%. The results obtained by Martin et al. [40] were in complete agreement with those obtained by the PM. Their proposed cutoff value of 2 μg/ml fully agrees with the MIC of previous study [36].

The microplate format offers many advantages: many DSTs can be performed at the same time, and the method is cost effective, including the reagents. It is also rapid and gives quantitative MIC results.

Umubyeyi et al. [49] reported susceptibility for 120 *M. tuberculosis* isolates susceptible to OFX by the BACTEC 460 method, the total of susceptible strains was 106. Of the 120 strains, 14 were resistant with the BACTEC 460 method, and had a MIC value of 4 μg/ml (8 isolates) and 8 μg/ml (6 isolates) using the REMA method. At 2 μg/ml of OFX, the specificity and sensitivity were both 100%. The colorimetric resazurin assay was found to be an alternative low-cost and rapid method to detect resistance to anti-tuberculosis drug resistance.

The contribution of TB laboratories worldwide, through rapid and accurate DST, is very important for the management of MDR TB, especially in low-income countries where most cases of MDR-TB occur.

In this study, the results were obtained after 8 days with the colorimetric method and are in nearly complete agreement with those obtained by the PM. In a quantitative test such as REMA, it is also important to establish which of the concentrations involved in the test will separate susceptible from resistant strains. The cutoff represents the “critical concentration” that defines susceptible and resistant strains based on the best fit of the colorimetric results with the conventional method.

Our proposed cut-off value for PAS and OFX was 2 μg/ml and for KAN was 2.5 μg/ml. This is in agreement with the MIC proposed by Martin et al. [40] and Umubyeyi et al. [49]. The tentative breakpoint concentration of ETH was 2.5 μg/ml which is in agreement with the MIC proposed by Martin et al. [36].

Advantages of the micro titer format are being faster, lower-cost and easy to perform and interpret, and does not need special equipment or reagent. The cost of the test will vary according to the setting and where it is used; however, it compares favorably with the conventional proportion method in LJ medium. One disadvantage, however, is biosafety, since the plates require the use of liquid medium and could generate aerosols. It has recently been shown that this format can be adapted for screw-cap tubes to avoid this situation [41,43,50]. 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 MDR TB 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 [51].

**Conclusion**

Resazurin test was found to be reliable, simple to perform for the rapid detection of anti-tuberculous drug resistance and economically inexpensive. It is potentially useful for low-resource countries. Therefore, it would be feasible to implement this method in laboratories with limited resources.
References


