Egyptian Journal of Medical Microbiology Volume 25 / No.3 / July 2016 89-94

ORIGINAL ARTICLE

Rapid colorimetric Resazurin Microtiter Assay (REMA) and MTT Assay for Testing susceptibility of *Mycobacterium tuberculosis* to Isoniazid and Rifampicin

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Key words:

REMA, MTT, Mycobacterium tuberculosis, MDR-TB, Isoniazid, Rifampicin

ABSTRACT

Background: *According to World Health Statistics, an incidence rate of 15 per 100,000 population of tuberculosis in Egypt was reported. Percentage of Multidrug Resistance (MDR) among new TB cases was 3.4%, while among previously treated TB cases was 15%. Several methods have been developed for the rapid detection of drug resistance tuberculosis compared with conventional time consuming drug susceptibility testing (DST). These methods include phenotypic assays which are easier to perform and shorten the turnout time for the diagnosis of MDR-TB.* ***Objective:*** *The aim of this study was to evaluate performance of two phenotypic methods for testing Mycobacterium tuberculosis susceptibility to Isoniazid (INH) and Rifampicin (RMP), with reference to the conventional proportion method (PM).* ***Methodology:*** *30 Mycobacterium tuberculosis isolates were tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) and resazurin microtiter assay (REMA), and compared with the results obtained with the gold standard methods: the proportion method on Lowenstein–Jensen medium.* ***Results:*** *Accuracy of 89.3% for INH and of 94.1% for RMP was observed using REMA assay, while accuracy of 96.9% for INH and of 93.2% for RMP was detected using MTT assay.* ***Conclusion:*** *MTT assay was noted to be more accurate than REMA assay. However, both assays are simple to perform for the rapid detection of Isoniazid and Rifampicin resistance and economically inexpensive. It is potentially useful for low-resource countries.*

INTRODUCTION

Tuberculosis (TB) now ranks as one of the leading causes of death. According to the WHO global tuberculosis report in 2015, 9.6 million people are estimated to have new TB infection in 2014. Worldwide, an estimated 3.3% of new TB cases and 20% of previously treated cases have multidrug-resistant TB (MDR-TB). Extensively drug-resistant TB (XDR-TB) had been recorded by 105 countries by 2015. About 9.7% of people with MDR-TB have XDR-TB. The number of reported cases of tuberculosis in Egypt according to World Health Statistics was 7, 467 new cases in 2014 with an incidence rate of 15 per 100,000 population. Fortunately, Egypt is still not on the WHO's list of 22 high-TB-burden countries or the 27 high MDR-TB burden countries. In Egypt, percentage of MDR among new TB cases was 3.4% while that among previously treated TB cases was 15% according to most recent year available data [1].

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In the last few years, several methods have been proposed for the rapid detection of drug resistance tuberculosis compared with time consuming conventional drug susceptibility testing (DST). These methods include phenotypic and genotypic techniques which are easier to perform and shorten the turnout time for the diagnosis of MDR-TB [2].

The aim of this study was to evaluate two phenotypic (colorimetric) methods; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) and resazurin microtiter assay (REMA) for *M. tuberculosis* susceptibility testing to the first-line drugs: Isoniazid (INH) and Rifampicin (RMP), with reference to the conventional proportion method (PM).

METHODOLOGY

Thirty *M. tuberculosis* isolates from Egyptian cases were obtained from the National Central Laboratory, Ministry of Health, Egypt. Susceptibility of *M. tuberculosis* was evaluated against 2 first-line anti-tuberculous drugs: isoniazid and rifampicin by 2 colorimetric methods; REMA and MTT assays. The results were compared to those obtained using the gold standard proportion method (PM).

1. Proportion method

PM was done as described by Canetti et al. [3] on LJ medium according to the standard procedure using the recommended critical concentration of 0.2 µg /ml for INH and 40 µg /ml for RIF.

2. Colorimetric methods reagents

INH and RMP were obtained from Sigma-Aldrich. Stock solutions at 1 mg/ml for INH, and 10 mg/ml for RMP were filter sterilized and stored at −20ºC. Preparation of working solutions was done 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). A stock solution of 0.02% (wt/vol) resazurin sodium salt powder (Sigma- Aldrich) was prepared in distilled water, sterilized by filtration, and stored at 4ºC for up to 1 week. A 5 mg/ml stock solution of MTT (Sigma–Aldrich) was prepared in PBS, pH 6.8, and was kept at 4ºC 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).

Isolates were refreshed by subculturing on LJ medium. The inoculum was prepared in 7H9-S broth, adjusted to McFarland standard tube no. 1, and further diluted 1:20 in 7H9-S broth for the test.

3. Resazurin microtitre assay

The REMA plate assay was carried out as described by Palmino et al. [4]. Hundred (100) µl of 7H9-S broth was dispensed in each well of a sterile flat-bottom 96-well plate, and serial twofold dilutions of each drug were prepared directly in the plate. Hundred (100) µl of inoculum was added to each well. A growth control and a sterile control were also included for each isolate. To avoid evaporation during the incubation, sterile water was added to all perimeter wells. The plate was covered and sealed in a plastic bag, then incubated at 37°C under a normal atmosphere. Thirty (30) µl of resazurin solution was added to each well after 7 days of incubation, and the plate was reincubated overnight. A change in color from blue to pink indicated the growth of bacteria (Figure 1). The drug concentration ranges used were 0.031 to 1 µg/ml for INH and 0.062–2.0 µg/ml for RMP. MIC was defined as the lowest concentration of drug that prevented this change in color. For INH, a strain is considered resistant if the MIC is greater than 0.25µg; for RIF, a strain is considered resistant if the MIC is greater than 0.5µg.

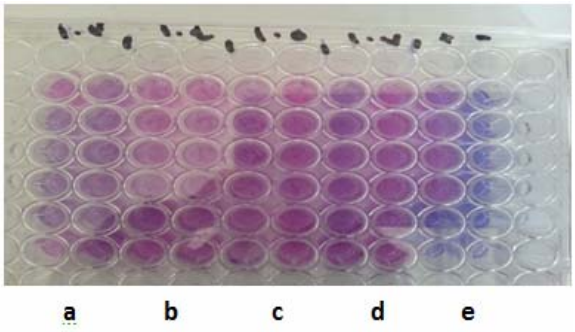

Fig. 1: REMA assay for 4 isolates: (a), (b), (c) and (d) isolates with MIC ›1µg/ml for INH, MIC ›2µg/ml for RMP (e) growth control

4. MTT assay

This method was done as described by Abate et al. [5]. The inoculum was prepared as described above for the REMA plate method, also using the same drugs' concentration range. Preparation of the 96-well plate was identical to the REMA plate assay. After 7 days incubation at 37ºC, 10 mL of the 5 mg/ml MTT solution was added to each well and the plate was reincubated overnight. On appearance of a violet precipitate (formazan) in the MTT well, 50 mL of the SDS/DMF solution was added to these wells and the plate reincubated for 3 h. A colour change from yellow to violet indicated the growth of bacteria (figure 2) and the MIC was interpreted as in the REMA plate assay.

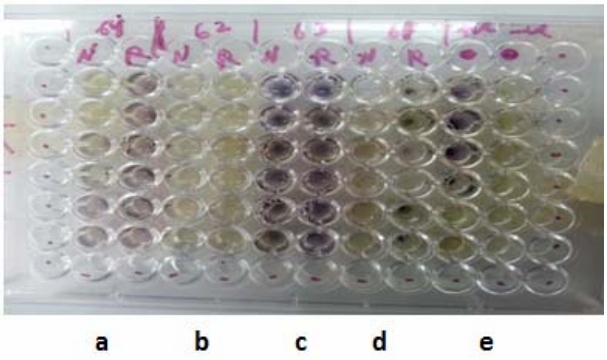

Fig. 2: MTT assay for 4 isolates: (a) isolate with MIC INH 0.5 µg/ml, MIC RMP ›2 µg/ml, (b) isolate with MIC‹0.03 µg/ml for INH, MIC‹0.06 µg/ml for RMP, (c) isolate with MIC ›lµg/ml for INH and MIC ›2 µg/ml for RMP (d) isolate with ‹0.03µg/ml for INH and MIC ›2 µg/ml for RMP (e) growth control

Statistical analysis

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 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)}, positive predictive value { the percentage of TP among the total of TP and FP}, negative predictive value {the percentage of TN among TN and FN}, 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 first-line drugs INH and RMP was performed for 30 *M. tuberculosis* isolates. Results of REMA and MTT were available after 8 days of incubation, while those of testing by the PM were available after 28-42 days. The DST results for the first-line drugs obtained by PM were as follows: 16 (53.3%) were resistant to INH and 14 (46.6%) were susceptible, while 17 (56.7%) were resistant to RMP and 13 (43.3%) were susceptible. For REMA and MTT assays; number and MICs for the 2 first-line drugs of isolates are shown in table 1.

Table 1 shows that MIC detected by REMA among INH resistant isolates (N = 19) was >1.0 µg/ml (18 isolates) and 0.5 µg/ml (1 isolate). Among RMP resistant isolates (N = 15), MIC was >2.0 µg/ml for the 15 isolates. Also it shows MIC detected by MTT and it was >1.0 µg/ml for 14 isolates, and 0.5 µg/ml for 1 isolate as regards INH. While, for RMP resistant isolates (N = 17), MIC was >2.0 µg/ml for 15 isolates and 2.0 µg/ml for 2 isolates.

Table 2 shows the DST results obtained by the REMA assay compared with the PM. For INH, 16 resistant isolates were detected by both methods. Among the remaining 14 isolates, 3 isolates gave a discrepant result as susceptible by PM while being resistant by REMA. For REMA agreement with PM regarding INH, it was 79.64%, and sensitivity of 100.0% and specificity of 78.6%. For RMP, both methods detected 13 susceptible isolates. As for the remaining 17 isolates, 2 isolates gave a discrepant result as susceptible by REMA while being resistant by PM. For REMA agreement with PM regarding RMP, it was 86.67%, and sensitivity of 88.2% and specificity of 100.0%.

DST results obtained by the MTT assay compared with the PM are shown in table 3. The agreement between the two tests for INH and RMP was found to be 93.33% and 86.43% respectively. The sensitivity of the MTT assay compared to that of PM was observed to be 93.8% and 94.1% for INH and RMP respectively. Specificities were 100% for INH and 92.3% for RMP.

Table 1: MICs of INH and RMP for *M. tuberculosis* isolates determined by REMA and MTT assays

Susceptibility results	*INH MIC (µg/ml) Number of isolates*						*RMP MIC (µg/ml) Number of isolates*						
	≤0.03	**0.06**	**0.25**	**0.5**	**1.0**	**>1.0**	**≤0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**>2.0**
REMA	7	2	2	1		18	12	1	1	1			15
MTT	13	2		1		14	7	2	4			2	15

*Breakpoint drug concentrations defining drug resistance is (>0.25µg/ml for INH and >0.5µg/ml for RMP)

Table 2: Comparison of results of DST of 30 *M. tuberculosis* isolates by PM and REMA assay

Drug	*PM*	*REMA* Number			*Agreement*	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*Accuracy*
		R	**S**	**Total**						
INH	**R**	16	0	16	79.64%	100.0%	78.6%	84.2%	100.0%	89.3%
	S	3	11	14						
	Total	19	11	30						
RMP	**R**	15	2	17	86.67%	88.2%	100.0%	100.0%	86.7%	94.1%
	S	0	13	13						
	Total	15	15	30						

Table 3: Comparison of results of DST of 30 *M. tuberculosis* isolates by PM and MTT assay

Drug	*PM*	*MTT*			*Agreement*	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*Accuracy*
		Number								
		R	S	Total						
INH	**R**	15	1	16	93.33%	93.8%	100.0%	100.0%	93.3%	96.9%
	S	0	14	14						
	Total	15	15	30						
RMP	**R**	16	1	17	86.43%	94.1%	92.3%	94.1%	92.3%	93.2%
	S	1	12	13						
	Total	17	13	30						

DISCUSSION

Drug resistant *M. tuberculosis* started to emerge after the use of the first anti-mycobacterial drugs, but the launch of both combination therapy and new more effective drugs seemed to be enough to control the disease. In fact, it was thought that TB could be eradicated by the end of 20th century [6]. However, by the mid-1990s, multi drug-resistant TB (MDR-TB) appeared in most countries. In 2006, extensively drug-resistant TB (XDR-TB) emerged [7]. Unfortunately the diagnosis of MDR-TB and XDR-TB is hindered due to lacking of effective rapid diagnostic tests for drug sensitivity. Rapid determination of drug resistance allows early treatment of the disease and can reduce morbidity, mortality and infectiousness [8]. However, alternative methods providing rapid detection of resistance to anti-tuberculous drugs were introduced. They are simple to perform and reduce the time to report first results compared to classical conventional methods [2].

The aim of this study was to evaluate two rapid low cost phenotypic techniques for the rapid identification of MDR-TB with reference to golden standard PM. The first technique was the REMA assay and the second one was the MTT assay. Thirty *M. tuberculosis* isolates isolated from Egyptian cases diagnosed as pulmonary TB were provided by the National Central Laboratory, Ministry of Health, Egypt.

REMA assay results of DST to INH showed that 19 (63.33%) isolates were resistant (MIC> 0.25µg/ml) and 11 (36.66%) isolates were sensitive (MIC ≤ 0.03µg/ml), and as for RMP 15 (50%) isolates were resistant (MIC> 0. 5µg/ml) and 15 (50%) were sensitive (MIC ≤ 0.25µg/ml). By comparing REMA assay results with those obtained by the PM, we found that testing INH using REMA assay yielded a sensitivity of 100.0%, and specificity was 78.6%, with 84.2% PPV, 100% NPV and 89.3% accuracy. The sensitivity, specificity, PPV, NPV and accuracy for RMP were 88.2%, 100%, 100%, 86.7% and 94.1% respectively.

Ang and colleagues, [9] evaluated REMA assay in a study on 110 *M. tuberculosis* isolates in Philippine. In their study, REMA showed 100% sensitivity and 91.7% specificity for both INH and RMP which were comparable to our results except for specificity to INH as we recorded a much lower percentage. Also, Rivoire et al. [10] conducted another study on 77 *M. tuberculosis* isolates in Madagascar and reported comparable PPV for susceptibility and resistance to RMP and comparable NPV for INH by the REMA assay. For INH the PPV and the NPV were 97.7% and 94.7% respectively, and for RMP the PPV and the NPV were 98% and 100% respectively. Our results matched those reported by Palmino et al.[4] for PPV of RMP and NPV of INH (100% each), whose study was on 80 clinical isolates of *M. tuberculosis* in Peru to evaluate the performance of the REMA assay. The PPV and the NPV to INH and RMP susceptibility testing by the REMA assay were 98.2% and 100% respectively for INH and both 100% for RMP.

In a study conducted in Egypt on 30 *M. tuberculosis* isolates provided from National Central Laboratory, Ministry of Health, the sensitivity, specificity, predictive values and accuracy of the REMA assay were 100% for both RIF and INH [11], and that was concordant with results reported by Martin et al. [12] when they performed a similar study in different Latin American countries. Nateche et al. [13] reported comparable results to theirs when they performed a similar study on 136 *M. tuberculosis* isolates in Algeria. The sensitivity of the REMA assay for INH and RMP was 100% and the specificity was 98.3% and 99.2% respectively.

Our study showed that the accuracy of the REMA assay as a drug susceptibility testing for INH and RMP was 89.3% and 94.1% respectively. However, our results were lower than those reported by Martin et al. [12] whose study proved that the accuracy of the REMA assay was 100% for both INH and RMP and Nateche et al. [13] whose study showed that the accuracy of the REMA assay for RMP and INH was 100% and 99% respectively.

Results reported previously by many investigators confirmed that the REMA assay is reliable and can be used as an alternative method for drug susceptibility testing for anti-tuberculous drugs especially in the low resource countries; however the discrepancy in the

REMA test performance reported in the current study can't jeopardize that postulate because of relatively small sample size. Further studies to be conducted at the same conditions are warranted to investigate such discrepancy.

For MTT assay, results of drug susceptibility testing to INH showed that 15 (50%) isolates were resistant and 15 (50%) isolates were sensitive, while 17 (56.66%) isolates were resistant and 13 (43.33%) were sensitive to RMP. By comparing the results of the present study with those obtained by the PM, we found that the sensitivity, specificity, PPV, NPV, and accuracy for INH were 93.8%, 100%, 100%, 93.3%, and 96.9% respectively , and the sensitivity, specificity, PPV, NPV, and accuracy for RMP were 94.1%, 92.3%, 94.1%, 92.3%, and 93.2% respectively.

Our results matched those reported by Foongladda and colleagues [14]. They found that The MTT method had specificity and sensitivity values of 92.3% and 99.5%, respectively, for INH susceptibility testing. Also our results were comparable to results of Abate et al. [5] for RMP, as they reported a sensitivity and specificity of 100% using MTT method. Montoro et al. [15] documented earlier close results to ours for both INH and RMP sensitivity and specificity (100%, 96.4% and 100%, 100% respectively).

Meanwhile, performance of MTT as showed in this study was higher than that reported previously by Raut et al. [16]. They reported sensitivity, specificity, PPV, NPV, and accuracy for INH of 95.2%, 87.5%, 97.5%, and 77.7% respectively, and 86.8%, 100%, 100%, and 70.5% for RMP respectively.

REMA and MTT assay are rapid tests; turnaround time (TAT) takes only 8 days after culture on solid media versus 3-6 weeks for the susceptibility testing on LJ media by the PM. Although this TAT is similar to that reported by the BACTEC TB-460 system, however, the main disadvantage of the BACTEC TB-460 system is the radioactivity, requiring special disposal conditions. Other disadvantages are the costly machine and the cost of the imported culture media vials that limit the use of BACTEC TB-460 in middle or low-income countries [12]. REMA and MTT assay can be done using 96 well microtiter plates, so they have the advantage of screening many isolates at the same time. Also they are economical as do not need special instruments for reading the results, just a good eye to see the change in colour. These conclusions are supported by many previous studies [9, 10].

When comparing MTT versus REMA, it was found that the interpretation of colour change seems to be easier with the MTT assay, as its colour shift was much sharper; also the results of MTT assay were more accurate than those of REMA for INH (96.9% versus 89.3%) while comparable for RMP. On the other hand, resazurin is cheaper and doesn't precipitate as MTT, avoiding a further step of solubilisation in the test and less manipulation of the plate.

Biosafety in these methods is a concern, as the plates need to be opened after several days of incubation for the addition of the indicator dye, which carries a risk to the laboratory personnel due to the use of liquid medium which is prone to aerosols generation [17]. To avoid this risk, some studies have been performed using tubes instead of microtiter plates, which also gave comparable results but more expensive and time consuming than working with microtiter plates [15]. Another limitation of these tests is its subjectivity in reading color changes. Also, there is variety of protocols used in the studies, so more standardization is needed for these assays [8].

CONCLUSION

MTT test was found to be more accurate than REMA test. However, they are simple to perform for the rapid detection of anti-tuberculous drug resistance and economically inexpensive. They are potentially useful for low-resource countries.

Source of Funding: None.
Conflict of Interest: None.

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