Application of the resazurin microtitre assay for the detection of isoniazid and/or rifampicin resistant *Mycobacterium tuberculosis* clinical isolates in Central Vietnam

Nguyen Thi Binh Nguyen[1], Paola Molicotti[2], Tran Hung[1], Le Thanh Phuc[3], Nguyen Thi Kieu Diem[3], Nguyen Tan Chum[3], Le Trong Thach[4], Truong Van Hue[4], Ngo Viet Quynh Tram[5]

(1) Infectious diseases and Tuberculosis Dept., Hue University of Medicine and Pharmacy, Hue University, Vietnam

(2) Department of Biomedical Science, Microbiology and clinical Microbiology, University of Sassari, Italy

(3) Da Nang Lung Hospital, Vietnam

(4) Central Hospital 71, Thanh Hoa province, Vietnam

(5) Microbiology Department, Hue University of Medicine and Pharmacy, Hue University, Vietnam

Abstract

Background: Drug resistant tuberculosis (DR-TB) remains a global health problem. The diagnosis, treatment, and management of DR-TB are the major challenges to Vietnam National Tuberculosis Control Program. One of the most important solutions for overcoming this problem is developing reliable and low-cost methods, which have been proposed to Drug Susceptibility Testing (DST) to detect drug resistant tuberculosis. The methods have to be reasonably simple so that they can be widely used in provincial Lung hospitals. Thus, our team conducted this study with the aim to apply the Resazurin Microtiter Assay (REMA) as drug susceptibility testing for detecting rate of phenotyphic isoniazid- (INH) and/or rifampicin- (RIF) resistance of *Mycobacterium tuberculosis (MTB)* isolates in central Vietnam. **Method:** A total of 196 *Mycobacterium tuberculosis* clinical isolates were tested by the REMA and the results were compared with those of BACTEC MGIT 960 system. The REMA was performed in 96-well plates with the concentration of INH and RIF 1.00–0.031 µg/ml for INH and 2.00– 0.061 µg/ml, respectively. A strain is considered resistant to INH if the MIC is ≥ 0.25 µg/ml. A strain is considered resistant to RIF if the MIC is ≥ 0.5 µg/ml. **Results:** The REMA results showed 42 (21.42%) *MTB* isolates resistant to INH, 13(6.63%) *MTB* isolates resistant to RIF, among them there were 12 (6.61%) isolates resistant to both RIF and INH, which were categorized as multi-drug resistant tuberculosis (MDR-TB). The excellent results of REMA were compared to those of the BACTEC MGIT 960 as the standard method, the sensitivities for isoniazid and rifampicin were 100% for both, the specificities for isoniazid and rifampicin were 99.35%, 98.92%, respectively. The positive predictive value (PPV) and the negative predictive value (NPV) for INH resistance were respectively 100% and 99.49%. The PPV and NPV were respectively 100% and 98.98% for RIF. The accuracies were 99.49% and 98.98% for INH and RIF, respectively. The REMA plate method had the sensitivity of 100% and the specificity of 99.46%, and PPV and NPV of respectively 91.67% and 100% for the identification of MDR-TB strains. **Conclusion:** Resazurin microtiter assay appears to be a good alternative method for the determination of drug susceptibility testing in low-resource countries such as Vietnam because this method is simple, reliable and inexpensive.

Key words: *Tuberculosis, Mycobacterium tuberculosis, Resazurin Microtiter Assay, drug resistance.*

1. INTRODUCTION

Tuberculosis (TB) is an old infectious disease, caused by *Mycobacterium tuberculosis (MTB)*. However, the threat of tuberculosis to the public's health is increasing [1]. According to the World Health Organization (WHO) in 2018, 10 million patients with TB and 1.5 million deaths were attributed to the disease [2]. The human immunodeficiency virus pandemic, multidrug-resistant TB (MDR-TB), and extensive drug TB (XDR-TB) have emerged as major obstructions in the treatment and efficient control of disease, especially in underdeveloped and developing countries [3]. The early and accurate detection of drug resistance *MTB* plays an important role on using of appropriate treatment regimens for the patient and preventing the spread of DR-TB isolates in the population. The standard agar proportion methods used for

Corresponding author: Nguyen Thi Binh Nguyen, email: ntbnguyen@huemed-univ.edu.vn
Ngo Viet Quynh Tram, email: nvqtram@huemed-univ.edu.vn
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drug susceptibility testing (DST) is low-cost, but time-consuming. In recent years, the commercial liquid medium BACTEC 460-TB, BACTEC MGIT 960, or molecular methods for rapid identification of drug resistant have reduced the turn-around time. However, these methods require costly reagents, modern equipment, and high-skilled performers, which are not affordable for routine implementation in low-income countries [4], [5]. In 2018, Vietnam had 3126 laboratory-confirmed cases - MDR/RR-TB [2]. Currently, Vietnam is in the 13th position among the 30 countries with the most cases of drug-resistant TB (DR-TB) prevalence. The diagnosis, treatment, and management of DR-TB are the major challenges to Vietnam National Tuberculosis Control Program. The BACTEC GMIT 960 is utilized in the detection of *MTB* at many provincial Lung hospitals, but drug susceptibility testing of this system are only carried out some laboratories in Vietnam. The REMA plate method, developed by Martin and Palomino (2002), using the colorimetric indicator resazurin has been proposed for drug susceptibility testing of *M. tuberculosis* [6]. This method was endorsed by WHO for improving diagnosis of drug resistant tuberculosis in developing countries. Moreover, the resazurin microtiter assay (REMA) plate method has also been used successfully for determination of the minimum inhibitory concentration (MIC) with *MTB* clinical isolates, which assists clinical doctors to choose suitable regimens [6], [7], [8]. This study was applied the REMA for detecting rate of phenotyphic isoniazid- (INH) and/or rifampicin- (RIF) resistance of *Mycobacterium tuberculosis* isolates in central Vietnam and the results obtained were compared with those using the BACTEC MGIT 960 system (Becton Dickinson, Sparks, Md, USA).

2. MATERIALS AND METHODS

M. tuberculosis clinical isolates from 196 patients at Da Nang Lung Hospital and Central Hospital 71, Thanh Hoa province from June 2019 to June 2020. The isolates were identified as M. tuberculosis by the BACTEC MGIT 960 system. Mycobacterium tuberculosis strain H37Rv (ATCC 27294) was considered as the susceptible control strain. Multidrug-resistant TB strain identified previously from the Clinical Microbiological Department at Da Nang Lung Hospital was used as the resistance control strain.

Drugs

Drugs were obtained from HiMedia Laboratories Pvt.Ltd (India) in the powder. Isoniazid (INH) was diluted in water to concentration of 1 mg/ml and used as the stock solution. Stock of Rifampicin (RIF) was prepared in methanol at concentration of 10 mg/ml, filter sterilized and stored at -20oC until use.

Resazurin reagent

The resazurin sodium salt powder (Acros Organic NV) was prepared at a concentration of 0.02% in distilled water and be stored at 4 °C for 1 week.

Culture medium

The resazurin microtiter assay plate method was performed in 7H9-S medium containing Middlebrook 7H9 broth with 0.1% Casitone, 0.5% glycerol and 10% Oleic acid, Albumin, Dextrose, and Catalase (OADC) supplement (Becton Dickinson)[3].

Resazurin microtiter assay (REMA)

The first method used to determined susceptibility was REMA by following standard protocols of Palomino [6],[9],[10], [12]:

- 100 µl 7H9-S medium were dispensed in each well of a sterile 96-well flat bottom plate (Corning).

- Two-fold serial dilutions of individual drug were prepared directly on the plate by adding 100µl of the working solution of each drug to achieve the final concentrations. Range of tested drug concentrations was 1.00 – 0.031 µg/ml for INH and 2.00 – 0.061 µg/ml for RIF.

- The inoculum (100 µl/well) was prepared from the mycobacterial growth in MGIT, was adjusted to the 1.0 McFarland standards and diluted 1:20 in 7H9-S Medium

- A growth control containing no antibiotic and a sterility control without inoculation were also included in per isolate.

- 200 µl of sterile water was added to all perimeter wells to prevent evaporation during the incubation.

- The plates were covered, sealed by plastic bags, and incubated at 37 °C. After 7 days of incubation, 30 µl of resazurin solution was added to each well, incubated for 48 hours at 37 °C, and assessed for colour development. The level of colour change (blue to pink) indicates level of resazurin reduction which is correlated to bacterial growth.

- The MIC was defined as the lowest drug concentration that prevented this colour change (inhibited bacterial growth). 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; For RIF, a strain is considered resistant if the MIC is ≥ 0.5 µg/ml.

The BACTEC MGIT 960

The results of REMA were compared with the reference method using a liquid culture, the BACTECTM MGITTM 960 Mycobacterial Detection

System to confirm remaining susceptible or resistant, independently of the MIC. The BACTEC MGIT 960 was performed according to manufacturer protocol at the Da Nang Lung Hospital. The utilizing commercial kits were supplied with fixed concentrations of 0.1 µg/ml, 1.0 µg/ml, for INH, RIF, respectively [12], [13].

Statistical Analysis

Meta-analysis was performed by using Excel 2010 and MedCalc statistical software 2020.

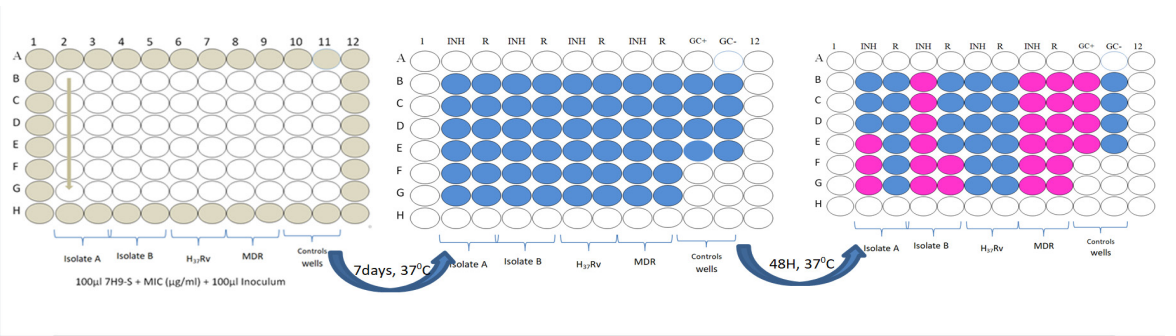

Figure 1. The REMA plate method

3. RESULTS

The REMA results confirmed 196 isolates containing *M. tuberculosis*. Number of isolates determined to be susceptible with INH was 154 (78.58%) which had the MIC of ≤ 0.125 mg/ml or lower. The remaining (21.42%) isolates determined to be resistant to INH with the MIC ≥ 0.25 mg/ml (Table 1).

Table 1. MICs of INH for 196 M. tuberculosis isolates determined by using the REMA plate method

MICs of INH for 196 *M. tuberculosis* isolates determined by REMA						
REMA	No. of isolates for which MIC(µg/ml) of INH was determined					
Resistance (n=42)	≤0.031	0.062	0.125	**0.25** 11 (5.61%)	**0.5** 10 (5.10%)	**≥ 1** 21 (10.71%)
Susceptible (n=154)	76 (38.78%)	46 (23.47%)	32 (16.33%)			

The REMA results also determined 183(93.34%) isolates which were susceptible to RIF with MIC of ≤ 0.25 µg/ml and 13(6.63%) isolates showed an intermediate resistance with MIC ≥ 0.5µg/ml (table 2). Among them, 12 (6.61%) isolates resistant to both RIF and INH were categorized as Multi-Drug Resistant (MDR) strains (Table 3).

Table 2. MICs of RIF for 196 M. tuberculosis isolates determined by using the REMA plate method

MICs of RIF for 196 *M. tuberculosis* isolates determined by REMA						
REMA	No. of isolates for which MIC(µg/ml) of RIF was determined					
Resistance (n=13)	≤0.062	0.125	0.25	**0.5** 5 (2.55%)	**1** 2 (1.02%)	**≥ 2** 6 (3.06%)
Susceptible (n=183)	166 (84.69%)	12 (6.62%)	5 (2.55%)			

Table 3. The proportion of TB isolates resistant to TB drugs

REMA	N	(%)	95% CI
INH	42	21.42	18.64-26.23
RIF	13	6.63	4.54-9.13
RH	12	6.61	4.36-8.89

The results obtained by REMA and BACTEC MGIT 960 are compared in Table 4. For isoniazid, 41 isolates were found resistant and 154 susceptible by both methods; one isolates was susceptible by the BACTEC MGIT 960 but resistant by REMA. For rifampicin, 11 isolates were resistant and 185 susceptible by both methods; two isolates were susceptible by the BACTEC MGIT but resistant by REMA. The sensitivities for both isoniazid and rifampicin were the same (100%) and the specificities were 99.35 %, 98.92%, respectively. The positive predictive value (PPV) and negative predictive value (NPV) for INH resistance were respectively 100% and 99.49%. The PPV and NPV were respectively 100% and 98.98% for RIF. The overall concordances were 99.49% and 98.98% for INH and RIF, respectively. With reference to the BACTEC MGIT as the standard method, the REMA plate method had the sensitivity of 100% and the specificity of 99.46%, and PPV and NPV of respectively 91.67% and 100% for the identification of MDR-TB strains.

Table 4. Comparing the results of REMA with those of reference method

REMA	BACTEC MGIT 960		Se (%)	Sp (%)	PPV (%)	NPV (%)	Accuracy (%)
	Resistant	Susceptible					
INH Resistant	41	01	100	99.35	97.62	100	99.49
Susceptible	0	154					
RIF Resistant	11	02	100	98,92	84.62	100	98.98
Susceptible	0	183					
RH MDR	11	01	100	99.46	91.67	100	99.49
Non- MDR	0	184					

4. DISCUSSION

Many developing countries have serious difficulties for obtaining drug susceptibility information on MTB isolates due to financial or technical constraints. One of the most important measures for overcoming this issue is selecting and applying the appropriate methods for the detection of DR-TB. The proportion method on LJ medium requires about 4-8 weeks to have results. Drug susceptibility testing in liquid culture such as BACTEC 460 or GMIT 960 and other molecular methods are expensive and require specific equipment, so they are impractical for routine testing [14], [15]. After developed by Palomino et al 2002, many studies applied REMA for detecting DR-TB and showed a good correlation with the conventional proportion method [9], [11], [15]. Owing to its simplicity, reliability and low cost in comparison with other non-commercial DST, WHO recommended using REMA plate method in countries with limited resources like Vietnam [16], [17], [18].

This was the first time REMA used for testing drug susceptibility of MTB and the results were compared with those of BACTEC MGIT 960 method. In this study, the results of REMA plate method are highly sensitivity of 100%, 100% and specificity of 99.35%, 98.92% for INH and RIF, respectively. Similar results of REMA for sensitivity (100%), and specificity (96%-98.92%) in the literatures of Palomino et al., 2002, Montoro et al., 2005 and Miyata et al.,2013 [6], [11], [15].

The advantage of DST by REMA is its ability to determine the resistance level assessed through MIC value. The MICs play an important role on classification of drug resistant isolates by helping clinical doctors to provide better decision in treatment for patients [6], [11], [16], [17]. Based on a sub-classification (Palomino et al., 2002) of resistant isolates by REMA, classification was made based on resistance level, with cut-off values of 1µg/mL and 2µg/ml for INH and RIF, respectively [6]. As result

of this sub-classification, the resistant isolates were divided into: 21 (50%) INH high-resistance and (50 %) INH low- resistance; 8 (61.54%) RIF high-resistance and 5 (38.46%) RIF low-resistance. These results were similar with those of author Miyata M et al.,2013 but lower than those reported by Montoro et al., 2005 [11], [14], [15].

In our study, the proportion of MDR-TB is 6.61% (5.6% MTB strain from new TB patients and 14.5% MTB strains from previous TB patients), while, according to WHO report 2018, the percentage of MDR-TB in Vietnam is 3.6% new cases and 17% previously treated cases in Vietnam [2], [17]. One MTB isolate was found to be resistant to rifampicin but sensitive to INH. Thus, most of the RIF-resistant MTB isolates in our study, were resistant to INH [6,19,20]. The PPV and NPV of REMA for MDR- TB and non MDR- TB were 91.67% and 100% respectively (Table 4). With excellent concordance with BACTEC MGIT 960, REMA method in our study has also shown high levels of agreement for INH, RIF and MDR-TB with 99.49%, 98.89% and 99.49%, respectively and our results were also similar to those of other studies with REMA performed by Coban AY et al 2006 and Miyata M et al 2013 [11], [13].

The minimum major equipment required for performing this test includes a level P2 biosafety cabinet, an aerosol-contained centrifuge and a 37oC sterilized incubator in order to preventing risk of contamination. Therefore, high biosafety requirement of REMA is still a barrier to be applied widely, especially in health units with non-modern infrastructure [20].

5. CONCLUSION

The REMA plate method showed a high level of accuracy in DST compared with BACTEC MGIT 960. Because of its simplicity, reliability and low-cost, REMA is very potential to be applied in central laboratories in developing countries [21]. This method is useful for early detection of DR-TB isolates and to provide a better management as well as treatment for patients.

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