Tropical Biomedicine 33(4): 814–823 (2016)

Direct tetrazolium microplate assay (TEMA) for rapid drug susceptibility test screening of *Mycobacterium tuberculosis*

Wan Nor Amilah, W.A.W.[1*], Mohammad Lukman, Y.[1], Siti Suraiya, M.N.[2] and Noor Izani, N.J.[1]
[1]School of Health Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[2]Department of Medical Microbiology and Parasitology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[*]Corresponding author e-mail: dramilah@usm.my OR amilahwahab2015@gmail.com
Received 10 April 2016; received in revised form 9 June 2016; accepted 10 June 2016

Abstract. Rapid and inexpensive assays for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* (MTB) are urgently required especially in developing countries where tuberculosis cases are prevalent. In response to this necessity, a direct microplate-based colorimetric assay which excludes the use of pre-testing culture isolate was evaluated. MTB susceptibility to the first line anti-tuberculosis drugs was tested directly on sputum specimens using tetrazolium microplate assay (TEMA) method and the sensitivity, specificity, accuracy as well as mean turn-around time of TEMA were compared to the standard absolute concentration method (ACM). TEMA was performed on 41 acid fast bacilli (AFB) positive sputum specimens by direct inoculation of the processed specimens into the microplate wells containing serial-diluted first line anti-tuberculosis drugs using tetrazolium dye as growth indicator. Indirect TEMA was performed on MTB isolates of the corresponding samples. The minimum inhibitory concentrations (MICs) of isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and streptomycin (SM) were obtained for direct and indirect TEMA with reference to the absolute concentration method (ACM). After establishing the breakpoint MIC of each drug using receiver operating characteristics (ROC) curve, reliable results from direct TEMA were obtained for INH and SM, with excellent levels of sensitivity, specificity, and accuracy (more than 90%). The predictive values for susceptibility were 100% for INH, EMB and SM as well as 96% for RMP. A shorter mean turn-around time of 14 days was observed for direct TEMA ($P < 0.05$). Thus direct TEMA is potentially rapid, reliable and inexpensive DST screening method of MTB in countries with high prevalence rates of drug resistance tuberculosis.

INTRODUCTION

Mycobacterium tuberculosis (MTB) is an infectious agent responsible for tuberculosis (TB) in humans. In recent years TB has emerged or re-emerged as an important public health problem worldwide. Most cases occur in Asia and Africa with an estimated 8.6 million new cases worldwide in 2012 (WHO, 2013). South-East Asia Region accounts for 33% of all cases globally (WHO, 2009). There have been a steady increase in the number of TB cases in Malaysia with about 64.7 cases per 100,000 population in 2000 (Iyawoo, 2004) to about 72.4 cases per 100,000 population in 2011 (Ministry of Health Malaysia, 2011).

Currently, the development of multi-drug resistant tuberculosis (MDR-TB) and the emergence of extensively drug resistant tuberculosis (XDR-TB) posed major obstacle in the treatment and control of TB worldwide. An estimated 420,000 MDR-TB cases were reported worldwide with resistance to anti-TB drugs varied from one country to another and in different regions within the same country (Zignol *et al.*, 2006; WHO, 2008).

A study in Malaysia in the late 80's reported that the rate of primary resistance to any type of first line anti-TB drug was 13-15% while about 1% was multi-drug resistant (Jalleh *et al*., 1993). MDR-TB prevalence in Malaysia was 0.1% in 1997 (WHO, 2000). The latest report by WHO stated that total confirmed cases of MDR-TB in Malaysia was 74 cases in 2012 (WHO, 2013). However, to our knowledge, regular and systematic data on the prevalence of drug resistant MTB are not readily available in every state of the country. The reason for the paucity of information on MTB resistant pattern was the lack of a simple, quick and affordable technique for determining the antibiotic susceptibility in most laboratories.

Centers for Disease Control (CDC) have recommended simultaneous *in vitro* detection of MTB and drug susceptibility test (DST) because of the increased incidence of MDR-TB (CDC, 1993). The DST must be repeated if patient's culture remains positive after three months of treatment. The existing conventional DST methods namely absolute concentration method (ACM) and proportion method are fairly well standardized with clinical samples at least for the major anti-TB drugs (Goodwin, 2007). ACM is still utilized in some developing countries including Malaysia as reference DST of MTB even though the long turnaround time (TAT) of DST results displease physicians for the purpose of case management.

Numerous new techniques have been developed, aiming to detect growth inhibition as early as possible such as BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system, BACTEC 460 system, E-test, and molecular tools such as real-time PCR techniques or line-probe assay. However, these rapid DST methods are expensive and inappropriate for underprivileged setting especially in the low resource countries (Franzblau *et al*., 1998). One of WHO recommended PCR assay is Xpert MTB/RIF, an automated method for detecting and amplifying rifampicin resistance nucleic acids has improved many of limitation from other genotypic assays. Running the assay requires less laboratory facilities; user training and supply chain management because the device is self-enclosed. It has shown high sensitivity and specificity when tested with smear positive sputum however; this test cannot detect isoniazid (INH) resistance (Wilson, 2011; Wilson, 2013).

DST may either be performed by indirect or direct methods. In direct DST methods, concentrated smear positive specimens are inoculated directly into control and drug containing media whereas in indirect method, pure culture must be obtained prior to its inoculation. The direct DST methods including microscopic-observation drug susceptibility (MODS) and nitrate-reduction assays (NRA) have been established as the low cost, easy-to-perform alternatives for low-income countries (Musa *et al.*, 2005; Moore *et al.*, 2006; Affolabi *et al.*, 2008; Limaye *et al.*, 2010). Another major advantage of direct DST over the indirect method is shorter TAT but it is less standardized and gives high chance for contamination (Forbes *et al.*, 2002; Ramachandran and Paramasivan, 2003).

Recently, an inexpensive and rapid indirect colorimetric method for the determination of antibiotic susceptibility of MTB has been reported using various dyes such as alamar blue or resazurin (Yajko *et al.*, 1995; Franzblau *et al.*, 1998; Palomino *et al.*, 2002; Miyata *et al*., 2013), dimethylthiazol-diphenyltetrazolium bromide (MTT) (Gomez-Flores *et al.*, 1995; Martin *et al*., 2005; Raut *et al*., 2008) and 2,3,5-triphenyltetrazolium chloride (TTC) (Caviedes *et al*., 2002; Mohammadzadeh *et al*., 2006). This method is able to detect MTB susceptibility to the first line anti-tuberculosis drugs within 8 days post-culture using oxidation-reduction dyes and multiple samples can be handled at a time. However, the requirement for initial culture isolation of MTB from the clinical samples prior to the colorimetric assay results in longer laboratory turn-around time for the final sensitivity result. Thus, this study aimed to develop and evaluate the direct MTB susceptibility testing towards the first line anti-TB drugs in smear positive sputum using tetrazolium microplate assay (TEMA).

MATERIALS AND METHODS

Sample collection and processing

A total of 41 acid fast bacilli (AFB) positive sputum samples were collected from the Hospital Universiti Sains Malaysia (HUSM) and Hospital Raja Perempuan Zainab II (HRPZ II) in Kelantan as well as from Institut Perubatan Respiratori (IPR) in Kuala Lumpur, Malaysia over the period of 18 months. Ethical approval for the collection of sputum specimens in the study was obtained from the Human Research Ethics Committee, USM (USMKK/PPP/JEPeM [234.4.(1.5)]). AFB positive sputum specimens were processed for decontamination by the standard N-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) method within 24h. Decontaminated sputum samples were aliquoted and processed for inoculation onto Ogawa medium and drug sensitivity testing by TEMA method. The MTB culture isolates obtained from each sputum specimen were identified using DNA probe test (GenProbe, Inc San Diego, California). The isolates were used to test for susceptibility of MTB to the first line anti-TB drugs by ACM as the gold standard and also by indirect TEMA method. The reference strain H37Rv (ATTC 27294) was used as the reference susceptible strain.

Drug susceptibility test using direct and indirect TEMA

The anti-TB drugs, isoniazid (INH) (Sigma-Aldrich), ethambutol (EMB) (Sigma-Aldrich) and streptomycin (SM) (Sigma-Aldrich) were prepared in distilled water while rifampicin (RMP) (Sigma-Aldrich) was prepared in methanol at four time (4x) the final working concentration. All antibiotics stock solutions were filter-sterilized and stored at -20°C prior to use. The Middlebrook 7H9-S medium (Becton-Dickinson) containing Middlebrook broth, 0.1% (w/v), casitone, 0.5% (v/v), glycerol and supplemented with 10% (v/v) oleic acid, albumin, dextrose and catalase (OADC) (Becton-Dickinson) was prepared as recommended by the manufacturer. The tetrazolium-tween 80 mixture (1:1) was prepared with 1 part of tetrazolium (Sigma-Aldrich) at dilution of 1 mg/mL in absolute ethanol and 1 part of 10% tween 80 (Merck). TEMA was carried out as described previously by Franzblau *et al.* (1998) and Caviedes *et al.* (2002).

For direct TEMA, optimization has been initially performed in which the minimum inhibitory concentrations (MICs) of INH, RMP, EMB and SM were pre-determined using MTB H37Rv (ATCC 27294) spikes in negative sputum samples. Then, the aliquoted processed AFB-positive sputum specimens were directly inoculated into drug-free as well as serial dilutions of drug-containing Middlebrook 7H9-S broth media in the 96-well microtitre plate. The final drug concentration ranges were 0.0313 to 0.002 µg/mL for INH; 0.0313 to 0.002 µg/mL for RMP; 0.5 to 0.0313 µg/mL for EMB and 0.25 to 0.0156 µg/mL for SM.

For indirect TEMA, the bacterial suspensions (3×10^7 CFU/mL) were prepared from culture growth of the same specimens and subsequently inoculated into broth-containing microtitre plates as described for direct TEMA. The final drug concentration ranges were as follows: INH: 0.5 to 0.001 µg/mL; RMP: 0.25 to 0.0005 µg/mL; EMB: 8 to 0.0156 µg/mL and SM: 4 to 0.0078 µg/mL. Both direct and indirect TEMA were performed in duplicate. Wells containing broth media and bacterial suspension served as positive control while the wells containing only broth media as negative control. Parafilm sealed plates were incubated at 37°C for 5 days. Then, 50µl of fresh tetrazolium-tween 80 mixtures were added to wells at different time intervals at day 5 and reincubated for 24 hours. The color changes in positive control wells were observed to determine the subsequent addition of tetrazolium dye in the test wells. The MIC was defined as the lowest concentration of drug that prevented a change of colour of the tetrazolium in the test wells.

Drug susceptibility test using absolute concentration method (ACM)

The ACM was performed on Lowenstein-Jensen (LJ) medium according to the Malaysian National Public Health Laboratory Standard Procedure (WHO/TB/Techn. Guide/67.7). In brief, the LJ media were incorporated with drugs: 5 µg/mL INH, 20 µg/

mL SM, 50 µg/mL RMP and 3 µg/mL EMB. The drug-incorporated media were inspissated at 80°C for 50 minutes. The media were inoculated with the standardized inoculum (2×10^3 CFU/mL) from pure culture isolate. Then, LJ medium were incubated at 37°C in the presence of 5-10% CO_2 until growth was observed in the drug free LJ medium. H37Rv strain of MTB was used as a standard reference (control). Strains showing growth of greater than 1% or more than 20 colonies were classified as resistant. For periodic calibration, the drug concentrations were prepared in series and MICs were determined. The MIC was defined as the lowest drug concentration that reduced the growth of the test organism to 1% or less when compared with the control slope (less than 20 colonies). The concentration where the majority of strains were sensitive was taken as the MIC.

Data analysis

The MIC values obtained by direct and indirect TEMA were compared with the results of ACM. For the purpose of distinguishing between resistant and susceptible MTB strains, the breakpoint MIC for each drug was calculated based on the receiver operating characteristics (ROC) curve analysis using the Stata/SE 11.0. The best breakpoint MIC value of each drug was selected by maximizing the specificity which corresponded to small x-axis value (1 – specificity) and the sensitivity which corresponded to high y-axis value (sensitivity) in the ROC graph (corresponded to a point nearest to the upper left corner of the ROC graph).

An analysis was performed to determine whether the breakpoint MIC values based on ROC curve analysis defined for each method influenced the outcome of the DST results by determining the mean area under the curve (AUC) value for direct and indirect TEMA methods. The mean AUC of 1 was interpreted as perfect separation between the susceptible and the resistant strains while the mean AUC value of 0.5 or lower indicated that the susceptibility of the strains was indistinguishable. The MTB isolates with drug MIC values that equal to or more than drug breakpoint MIC were considered resistant whereas those with drug MIC values less than the drug breakpoint MIC were considered susceptible.

The sensitivity, specificity, accuracy, predictive value for susceptible (PVS) and predictive value for resistance (PVR) of direct and indirect TEMA of the clinical samples in comparison to ACM were calculated using the formula as shown below:

$$\text{Sensitivity} = \frac{\text{true resistant}}{\text{true resistant + false sensitive}} \times 100\%$$

$$\text{Specificity} = \frac{\text{true sensitive}}{\text{true sensitive + false resistant}} \times 100\%$$

$$\text{Accuracy} = \frac{\text{true resistant + true sensitive}}{\text{true resistant + false resistant + true sensitive + false sensitive}} \times 100\%$$

$$\text{PVS} = \frac{\text{true sensitive}}{\text{true sensitive + false sensitive}} \times 100\%$$

$$\text{PVR} = \frac{\text{true resistant}}{\text{true resistant + false resistant}} \times 100\%$$

The TAT was compared among the three DST methods and also among different smear-positive sputum samples used in direct TEMA method. TAT was defined as time taken from the date of samples processing to the date on which result was available. Comparison among all DST methods was performed using One-Way ANOVA and Kruskal-Wallis analysis using the Statistical Package for Social Science (SPSS) software version 20. Differences were considered significant at $P < 0.05$.

RESULTS

Out of 41 MTB isolates, 8 were found to be resistant to at least one drug and 33 isolates were susceptible to all drugs by ACM. The defined breakpoint MIC value for each drug

based on the ROC curve analysis using Stata Software for direct TEMA was 0.0313 µg/mL for INH, 0.0313 µg/mL for RMP, 0.5 µg/mL for EMB, and 0.25 µg/mL for SM and the defined breakpoint MIC value for indirect TEMA was 0.0625 µg/mL for INH, 0.0625 µg/mL for RMP, 1.0 µg/mL for EMB, and 4.0 µg/mL for SM. Table 1 shows the number of resistant and susceptible strains observed by the direct and indirect TEMA based on the defined breakpoint MIC compared to the results of the ACM. The sensitivity, specificity and accuracy values from direct and indirect TEMA methods compared to the results of ACM is shown in Table 2.

The direct TEMA showed good results for INH, EMB and SM with 100% sensitivity to all drugs with specificity of 92%, 55% and 97% and accuracy of 93%, 68% and 98% respectively. However, a lower sensitivity (75%) and specificity (68%) were observed for RMP. Meanwhile, the indirect TEMA showed very good result for INH with 100% sensitivity, specificity, and accuracy. However, the sensitivity, specificity and accuracy were lower for RMP, EMB and SM respectively. The results of PVS of all drugs were excellent in both direct and indirect TEMA (above 90%) and their PVRs were predictably low (Table 2).

Table 1. Susceptibility results of 41 MTB smear-positive sputum specimens obtained by direct and indirect TEMA methods in comparison to the absolute concentration method (ACM)

ACM	INH				RMP				EMB				SM			
	DT		IT		DT		IT		DT		IT		DT		IT	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
R	3	0	3	0	3	1	3	1	3	0	1	2	2	0	1	1
S	3	35	0	38	12	25	18	19	7	31	7	31	1	38	1	38
Total	6	35	3	38	15	26	21	20	10	31	8	33	3	38	2	39

DT: direct TEMA; IT: indirect TEMA; R: resistant; S: susceptible

Table 2. Sensitivity, specificity, accuracy, PVS and PVR of the direct and indirect TEMA of the first line anti-TB drugs

Methods	Drugs	Sensitivity[a] (%)	Specificity[b] (%)	Accuracy[c] (%)	PVS (%)	PVR (%)
Direct TEMA (n = 41)	INH	100	92	93	100	50
	RMP	75	68	68	96	20
	EMB	100	55	59	100	30
	SM	100	97	98	100	50
	Overall	94	78	80	99	38
Indirect TEMA (n= 41)	INH	100	100	100	100	100
	RMP	75	51	54	95	14
	EMB	33	82	78	94	13
	SM	50	97	95	97	50
	Overall	65	83	82	97	44

TEMA: tetrazolium microplate assay; PVS: predictive value for susceptible; PVR: predictive value for resistance; a; true resistant, b; true susceptible, c; the rate of correct results.

The AUC value for direct TEMA ranged from 0.713 to 0.974 (INH 0.961, RMP 0.713, EMB 0.776 and SM 0.974) while for indirect TEMA, the AUC value ranged from 0.570 to 1.0 (INH 1.0, RMP 0.635, EMB 0.570 and SM 0.930). The differences observed in the AUC results obtained by direct and indirect TEMA for the tested drugs were not significant (INH, $P = 0.075$; RMP, $P = 0.462$; EMB, $P = 0.406$; SM, $P = 0.499$).

The comparison of TAT for the three DST methods and the median TAT of direct TEMA with different smear-positive categories are shown in Table 3 and 4 respectively. By using the one-way ANOVA test followed by post-hoc multiple comparison test Dunnet C procedures, the differences between the mean TAT results obtained between ACM and direct TEMA, ACM and indirect TEMA, and direct TEMA and indirect TEMA methods were all significant ($p < 0.001$). The direct TEMA method showed shortest TAT (7 to 31 days; mean of 14.07 days) followed by indirect TEMA (36 to 44 days; mean of 37.15 days) and ACM (57 to 121 days; mean of 92.86 days) (Table 3).

A significant difference in median TAT was observed between the AFB 3+ smear category (9 days) with the AFB 2+ smear category (12.5 days; $p = 0.018$), AFB 1+ smear category (17.0 days; $p = 0.008$) and AFB scanty smear categories (18.0 days; $p = 0.035$) (Table 4). The difference in median TAT observed between other smear-positive categories pairs were not significant ($p > 0.05$). Thus, a shorter median TAT of 14 days can be achieved by using the AFB 3+ smear category in direct TEMA. However, comparison for indirect TEMA and ACM was not done as these methods used pure cultures.

Table 3. Comparison of mean turnaround time (TAT) between absolute concentration method (ACM), direct and indirect TEMA

	TAT[a] (days)		
	Mean	SD	Range
ACM	92.86	18.88	57–121
Direct TEMA	14.07	5.24	7–31
Indirect TEMA	37.15	1.59	36–44

[a]One-way ANOVA test was applied followed by Post-hoc multiple comparisons test Dunnet C procedures: the mean difference of turnaround time is significant between ACM and direct TEMA, ACM and indirect TEMA and between direct and indirect TEMA.
F (df) = 522.12 (2), $p < 0.001$

Table 4. The median turnaround time (TAT) of different smear-positive categories in direct TEMA

Smear-Positive Categories[b]	TAT[a] (days)	
	Median	IQR
Scanty	18	0
1+	17.0	5
2+	12.5	5
3+	9.0	6

[a]Kruskal Wallis test was applied followed by Post-hoc tests:
[b]Scanty, 1-9 AFB/100 fields; 1+, 10-99/100 fields; 2+, 1-10 AFB/field; 3+, more than 10 AFB/field
1-9 AFB vs 1+, $p = 0.583$; 1-9 AFB vs 2+, $p = 0.110$; 1-9 AFB vs 3+, $p = 0.035$; 1+ vs 2+, $p = 0.078$; 1+ vs 3+, $p = 0.008$; 2+ vs 3+, $p = 0.018$
χ^2 (df) = 12.269 (3), $p = 0.007$

DISCUSSION

In Malaysia, ACM is the standard reference method routinely performed by the designated Malaysian National Public Health Laboratory (NPHL) for mycobacterial DST performance. All samples in this study were cultured on the egg-based solid media and DST results by ACM were obtained from NPHL and analyzed. Although MGIT is one of the recommended method for use by WHO (WHO, 2007), however in NPHL, MGIT is only indicated when unreliable ACM results are obtained from the contaminated cultures and also when more accurate DST results are warranted for immunocompromised patients as well as those suspected with treatment failure.

In this study, the results obtained from direct TEMA method were partially encouraging but was relatively better compared to the indirect TEMA method. The sensitivity towards the INH was very good but unexpectedly it was not so for RMP. Unlike INH and RMP, drugs like EMB and

SM are considered as difficult drugs to test (Abate *et al*., 2004; Martin *et al*., 2005). Surprisingly, sensitivity and specificity towards SM was very good considering SM is difficult to test. The accuracy of the test for INH and SM was very good and showed similar results as in previous TEMA studies (Caviedes *et al*., 2002; Mohammadzadeh *et al*., 2006; Miyata *et al*., 2013). The PVR results of all drugs in both direct and indirect TEMA were predictably low as sample prevalence for resistant MTB strain was low due to low disease prevalence in the studied population.

For RMP and EMB drugs, previous findings gave moderate performance in sensitivity and specificity (Caviedes *et al*., 2002; Musa *et al*., 2005). The discordance might possibly be overcome by adjusting the tentative breakpoint concentration (cut-off values) and increasing the samples size of resistant strains (Musa *et al*., 2005). In this study, the cut-off points obtained were different compared to previous studies (Palomino *et al*., 2002; Martin *et al*., 2005; Martin *et al*., 2007; Brady *et al*., 2008; Miyata *et al*., 2013) even though similar method was employed. The differences might probably due to technical variations which may come from the physico-chemical labile environment of the test (Kim, 2005). Furthermore, the number of strains resistant to any two drugs found in this study was low. For indirect TEMA however, full agreement was seen only for INH but not with other drugs.

In this study, direct TEMA produced shorter TAT compared to the indirect TEMA and ACM. This was in concordance with previous studies that have been carried out using direct DST method such as MODS (Moore *et al*., 2006), MGIT assay (Goloubeva *et al*., 2001; Siddiqi *et al*., 2012), NRA (Musa *et al*., 2005; Affolabi *et al*., 2008; Visalakshi *et al*., 2010) and colorimetric assay (Abate *et al*., 2004; Boum *et al*., 2013). Furthermore, this study also measured the TAT by the degree of AFB positive smear. From our observation, TAT for direct TEMA with 3+ was significantly shorter than 2+, 1+ and scanty smear category. The mycobacterial count determines the grading of AFB positive smear as observed under the microscope. Therefore, higher numbers of mycobacterial from higher AFB smear grading will eventually produce better reaction than the lower one (Visalakshi *et al*., 2010; Siddiqi *et al*., 2012), thus supporting our finding. This study however did not measure the TAT by the degree of AFB positive smear for indirect TEMA and ACM because these two methods do not depend on AFB smear grading since they use isolated pure cultures. Most of the broth-based DST studies used pure isolates from the culture leading to longer TAT (Yajko *et al*., 1995; Franzblau *et al*., 1998; Mshana *et al*., 1998; Caviedes *et al*., 2002).

Unlike other direct DST methods, direct TEMA has several added advantages. The interpretation of colour changes with direct TEMA only requires visualization with naked eyes. Thus, it seems to be easier compared to MODS in using microscope and other colorimetric assay using MTT dye which are less sharper with poor colour shift (Martin *et al*., 2005). Direct TEMA can be performed on multiple samples while NRA utilizes tube format making it unsuitable for multiple samples handling. Direct DST method using MGIT is good however the instrument is expensive. Direct TEMA is inexpensive since no special equipment or reagents are needed. Most of consumables and reagents used are common and can be built in-house. Nevertheless, strict compliance with safety and protection measures is still mandatory as it involves handling of the viable, infectious bacterial suspensions. The procedure must be carried out in a laboratory that meets the WHO standards for biosafety level 2 with access restricted to authorized personnel only.

In conclusion, the determination of antibiotic susceptibility by using direct TEMA method is relatively simple and rapid in overcoming the limitation of having to acquire initial isolation of *Mycobacterium* and thus shorten the TAT for DST. Sensitivity and specificity can be further improved to produce reliable results by optimizing AFB smear positive degree and the breakpoint value for the tested drugs, as well as increase the number of resistant strains. Thus this new method is a potential tool to provide an alternative and efficient technique for DST to be used particularly in developing

countries with limited laboratory facilities and resources.

Acknowledgements. This study was supported by the Short Term Grant: 304/PPSK/61311023 from Universiti Sains Malaysia (USM). We gratefully acknowledge the staffs of the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia; Institute of Respiratory Medicine (IPR), Kuala Lumpur and Pathology Department (Microbiology Section) of Hospital Raja Perempuan Zainab II, Kelantan, Malaysia. Mohammad Lukman (co-author) was endowed with a USM Graduate Assistant Scheme from Universiti Sains Malaysia and MyBrain15 from Ministry of Education, Malaysia.

REFERENCES

Abate, G., Aseffa, A., Selassie, A., Goshu, S., Fekade, B., WoldeMeskal, D. & Miorner, H. (2004). Direct colorimetric assay for rapid detection of rifampin-resistant *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* **42**(2): 871-873.

Affolabi, D., Odoun, M., Sanoussi, N.D., Martin, A., Palomino, J.C., Kestens, L., Anagonou, S. & Portaels, F. (2008). Rapid and inexpensive detection of multi drug-resistant *Mycobacterium tuberculosis* with the nitrate reductase assay using liquid medium and direct application to sputum samples. *Journal of Clinical Microbiology* **46**(10): 3243-3245.

Boum, Y., Orikiriza, P., Rojas-Ponce, G., Riera-Montes, M., Atwine, D., Nansumba, M., Bazira, J., Tuyakira, E., De Beaudrap, P., Bonnet, M. & Page, A.L. (2013). Use of colorimetric culture methods for detection of *Mycobacterium tuberculosis* complex isolates from sputum samples in resource-limited settings. *Journal of Clinical Microbiology* **51**(7): 2273-9.

Brady, M.F., Coronel, J., Gilman, R.H. & Moore, D.A. (2008). The MODS method for diagnosis of tuberculosis and multidrug resistant tuberculosis. *Journal of Visualized Experiments* **(18)**: e845.

Caviedes, L., Delgado, J. & Gilman, R.H. (2002). Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* **40**(5): 1873-1874.

Centers for Disease Control (1993). Initial therapy for tuberculosis in the era of multidrug resistance: recommendations of the Advisory Council for the elimination of tuberculosis. *MMWR Morbidity Mortality Weekly Report* **42**: l-8.

Forbes, B.A., Sahm, D.F. & Weissfeld, A.S. (2002). *Bailey & Scott's Diagnostic Microbiology* (11 ed.). USA: Mosby.

Franzblau, S.G., Witzig, R.S., McLaughlin, J.C., Torres, P., Madico, G., Hernandez, A., Degnan, M.T., Cook, M.B., Quenzer, V.K., Ferguson, R.M. & Gilman, R.H. (1998). Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate alamar blue assay. *Journal of Clinical Microbiology* **36**(2): 362-366.

Goloubeva, V., Lecocq, M., Lassowsky, P., Matthys, F., Portaels, F. & Bastian, I. (2001). Evaluation of mycobacteria growth indicator tube for direct and indirect drug susceptibility testing of *Mycobacterium tuberculosis* from respiratory specimens in a Siberian Prison Hospital. *Journal of Clinical Microbiology* **39**(4): 1501-1505.

Gomez-Flores, R., Gupta, S., Tamez-Guerra, R. & Mehta, R.T. (1995). Determination of MICs for *Mycobacterium avium-M. intracellulare* complex in liquid medium by a colorimetric method. *Journal of Clinical Microbiology* **33**(7): 1842-1846.

Goodwin, A. (2007). *Mycobacterium tuberculosis* and other nontuberculous mycobacteria. In*:* Mahon, C.R., Lehman, D.C. and Manuselis, G. (eds.), *Textbook of Diagnostic Microbiology* Third ed. St. Louis, Mo.: Saunders Elsevier, pp 683-707.

Iyawoo, K. (2004). Tuberculosis in Malaysia: problems and prospect of treatment and control. *Tuberculosis* **84**(1): 4-7.

Jalleh, R.D., Kuppusamy, I., Soshila, R., Aziah, A.M. & Faridza, M.Y. (1993). A study of primary drug resistance in pulmonary tuberculosis in west Malaysia 1984-1987. *Medical Journal of Malaysia* **48**(2): 113-116.

Kim, S. (2005). Drug-susceptibility testing in tuberculosis: methods and reliability of results. *European Respiratory Journal* **25**(3): 564-569.

Limaye, K., Kanade, S., Nataraj, G. & Mehta, P. (2010). Utility of microscopic observation of drug susceptibility (MODS) assay for *Mycobacterium tuberculosis* in resource constrained settings. *The Indian Journal of Tuberculosis* **57**(4): 207-212.

Martin, A., Morcillo, N., Lemus, D., Montoro, E., da Silva Telles, M., Simboli, N., Pontino, M., Porras, T., León, C. & Velasco, M. (2005). Multicenter study of MTT and resazurin assays for testing susceptibility to first-line anti-tuberculosis drugs. *The International Journal of Tuberculosis and Lung Disease* **9**(8): 901-906.

Martin, A., Portaels, F. & Palomino, J.C. (2007). Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy* **59**(2): 175-183.

Ministry of Health Malaysia (2011). Public health: tuberculosis & leprosy prevention and control. Annual report Ministry of Health Malaysia 2011. p. 61.

Miyata, M., Pavan, F.R., Sato, D.N., Marino, L.B., Hirata, M.H., Cardoso, R.F., de Melo, F.A. & Leite, C.Q. (2013). Comparison of resazurin microtiter assay performance and BACTEC MGIT 960 in the susceptibility testing of Brazilian clinical isolates of *Mycobacterium tuberculosis* to four first-line drugs. *Brazilian Journal of Microbiology* **44**(1): 281-285.

Mohammadzadeh, A., Farnia, P., Ghazvini, K., Behdani, M., Rashed, T. & Ghanaat, J. (2006). Rapid and low-cost colorimetric method using 2,3,5-triphenyltetrazolium chloride for detection of multidrug-resistant *Mycobacterium tuberculosis*. *Journal of Medical Microbiology* **55**(12): 1657-1659.

Moore, D.A., Evans, C.A., Gilman, R.H., Caviedes, L., Coronel, J., Vivar, A., Sanchez, E., Piñedo, Y., Saravia, J.C. & Salazar, C. (2006). Microscopic-observation drug-susceptibility assay for the diagnosis of TB. *New England Journal of Medicine* **355**(15): 1539-1550.

Mshana, R.N., Tadesse, G., Abate, G. & Miorner, H. (1998). Use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide for rapid detection of rifampin-resistant *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* **36**(5): 1214-1219.

Musa, H.R., Ambroggi, M., Souto, A. & Ängeby, K.K. (2005). Drug susceptibility testing of *Mycobacterium tuberculosis* by a nitrate reductase assay applied directly on microscopy-positive sputum samples. *Journal of Clinical Microbiology* **43**(7): 3159-3161.

Palomino, J.-C., Martin, A., Camacho, M., Guerra, H., Swings, J. & Portaels, F. (2002). Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* **46**(8): 2720-2722.

Ramachandran, R. & Paramasivan, C. (2003). What is new in the diagnosis of tuberculosis? Part II: techniques for drug susceptibility testing. *Indian Journal of Tuberculosis* **50**(4): 197-202.

Raut, U., Narang, P., Mendiratta, D.K., Narang, R. & Deotale, V. (2008). Evaluation of rapid MTT tube method for detection of drug susceptibility of *Mycobacterium tuberculosis* to rifampicin and isoniazid. *Indian Journal of Medical Microbiology* **26**(3): 222-227.

Siddiqi, S., Ahmed, A., Asif, S., Behera, D., Javaid, M., Jani, J., Jyoti, A., Mahatre, R., Mahto, D. & Richter, E. (2012). Direct drug susceptibility testing of *Mycobacterium tuberculosis* for rapid detection of multidrug resistance using the Bactec MGIT 960 system: a multicenter study. *Journal of Clinical Microbiology* **50**(2): 435-440.

Visalakshi, P., Meharwal, S.K., Myneedu, V.P. & Behera, D. (2010). Evaluation of direct method of drug susceptibility testing of *Mycobacterium tuberculosis* to rifampicin and isoniazid by nitrate reductase assay in a national reference laboratory. *Diagnostic Microbiology and Infectious Disease* **66**(2): 148-152.

Wilson, M.L. (2011). Recent advances in the laboratory detection of *Mycobacterium tuberculosis* complex and drug resistance. *Clinical Infectious Diseases* **52**(11): 1350-1355.

Wilson, M.L. (2013). Rapid diagnosis of *Mycobacterium tuberculosis* infection and drug susceptibility testing. *Archives of Pathology and Laboratory Medicine* **137**(6): 812-819.

World Health Organization (2000). Anti-tuberculosis drug resistance in the world: Report no. 2; prevalence and trends; the WHO/IUATLD global project on anti-tuberculosis drug resistance surveillance: communicable diseases, World Health Organization.

World Health Organization (2007). Use of liquid TB culture and drug susceptibility testing (DST) in low and medium income settings. Summary report of the expert group meeting on the use of liquid culture media. WHO, Geneva, Switzerland.

World Health Organization (2008). Anti-tuberculosis drug resistance in the world, fourth global report: the WHO/IUATLD global project on anti-tuberculosis drug resistance surveillance: World Health Organization.

World Health Organization (2009). Global tuberculosis control: WHO report: global tuberculosis programme, World Health Organization.

World Health Organization (2013). Global tuberculosis report 2013.

Yajko, D.M., Madej, J.J., Lancaster, M.V., Sanders, C.A., Cawthon, V.L., Gee, B., Babst, A. & Hadley, W.K. (1995). Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* **33**(9): 2324-2327.

Zignol, M., Hosseini, M.S., Wright, A., Lambregts–van Weezenbeek, C., Nunn, P., Watt, C.J., Williams, B.G. & Dye, C. (2006). Global incidence of multidrug-resistant tuberculosis. *Journal of Infectious Diseases* **194**(4): 479-485.