The Hong Kong Tuberculosis, Chest and Heart Diseases Association
Scientific Committee on Lung Health
Final report

1. **Project No.:** TB project 1/2014

2. **Grant Period:** Commencement Date: March 2015 End Date: February 2016

3. **Title of Project:**

Optimizing MGIT pyrazinamide susceptibility testing using a reduced inoculum

4. **Principal Applicant:**

Dr. YAM Wing Cheong

5. **Administering Institution:**

Department of Microbiology, The University of Hong Kong

6. **Aims/ Objectives of the research:** List the main objectives as stated in the approved proposal.

Approved aims/ objectives	**Objectives / indicators achieved**
	Low 1 / 2 / 3 / 4 / High 5
Indicator 1: Estimating the proportion of pyrazinamide (PZA) resistance found by each testing method after completing paired tests in 30 culture isolates. This may help evaluate whether the estimated sample size is adequate.	5
Indicator 2: Estimating the proportion with failure to grow for each testing method after completing paired tests in 30 culture isolates. This may help fine tune the required sample size.	5

7. **Timetable of Work:** Document the study progress according to the proposed timetable.

This study involved eligible culture isolates of *M. tuberculosis* consecutively received by Queen Mary Hospital Microbiology Laboratory serving patients in Queen Mary Hospital and Graham Hospital with *M. tuberculosis* isolated since 1 Jun 2013. During the first month of study (March 2015), ordering of necessary reagents were completed and the recruited Research Assistant was trained to perform PZA susceptibility testing. For the first 30 culture isolates, complete concordance of PZA susceptibility testing result were exhibited by both standard and reduced inoculums but one of the 30 culture isolates failed to grow in the MGIT with reduced inoculums. The study finished with another 120 culture isolates that a total of 150 culture isolates were used for the comparative study. Another 21 achieved PZA resistant strains were also collected for the comparative study. Pyrazinamidase (PZase) activity and DNA sequencing of *pncA* gene were compared between PZA susceptible and resistant isolates.

8. **Summary of the project and its results:**

Among the 150 culture isolates, 11 isolates failed in the MGIT 960 PZA susceptibility test due to under inoculums size (3 isolates for both standard and reduced inoculums; 8 isolates for reduced inoculums only). The failure rate for standard and reduced inoculums was 1.8% (3/171) and 6.4% (11/171) respectively. Only 139 isolates were eligible for further study and analysis. Another 21

archived PZA resistant strains collected between 2003-2013 were also included for comparative study. However, one strain was later confirmed to be PZA susceptible that 140 PZA susceptible and 20 PZA resistant strains were used for the McNemar's analysis, indicating there is no significant difference between standard and reduced inoculums for MGIT960 PZA susceptibility test (p = 0.6171; Odd Ratio = 1; 95% CI: 0.072 – 13.796).

The 20 PZA resistant archived strains collected between 2003-2013 were further characterized by MGIT960 PZA (standard and reduced inoculums) test, PZase activity and DNA sequencing to identify *pncA* mutations. The most common PZA resistant mutations at pncA were G162D and I90S. Novel mutations including insertions and deletions were also detected among PZA resistant strains. PZase activity were negative for all PZA resistant strains while 20 randomly selected PZA susceptible strains were positive to PZase activity with no mutations in the *pncA* gene. One strain (WC036) reported as PZA susceptible by TB Reference laboratory (PHLSB) was confirmed resistant to PZA by MGIT960 (both standard and reduced inoculum) with *pncA* mutations and negative for PZase activity. Another strain (WC205) reported as susceptible to all first line drugs but resistant to PZA was also confirmed susceptible to PZA by MGIT960 (both standard and reduced inoculum) with wild type *pncA* and positive for PZase activity.

In this study, testing 160 *M. tuberculosis* isolates indicated no significant difference between standard and reduced inoculums for MGIT960 PZA susceptibility test (p >0.05). The failure rate for standard and reduced inoculums was 1.8% and 6.4% respectively. The 1 false susceptible and 1 false resistant isolates reported by TB reference Laboratory (PHLSB) was probably accounted by the drug susceptibility test other than MGIT960. Since 2014, PHLSB has adopted the MGIT960 system with standard inoculums size for routine service to all Hospital Authority Hospitals in Hong Kong. For routine PZA MGIT 960 susceptibility testing using standard inoculums, clinical isolates reported as resistant to PZA should be confirmed by PZase activity and *pncA* gene mutation analysis using PCR-sequencing.

Final Report

Date: 31 May, 2016.

A. Project Title : Optimizing MGIT pyrazinamide susceptibility testing using a reduced inoculum

Study Period: March 2015 – February 2016

Investigator: YAM Wing Cheong, Scientific Officer / Honorary Associate Professor

Administering Institute: Dept of Microbiology, The University of Hong Kong

Date of submission: 6 Nov., 2014.

B. Summary (300words):

Pyrazinamide (PZA) is considered an essential component of first-line TB therapy, it is important that laboratories should adopt a successful algorithm to provide rapid and accurate susceptibility results for PZA. In this study, testing 160 *M. tuberculosis* isolates indicated no significant difference between standard and reduced inoculums for MGIT960 PZA susceptibility test ($p > 0.05$). The failure rate was higher for reduced inoculums (6.9%) than the standard inoculums (1.8%).

Further investigation on isolates using PCR-sequencing indicated all PZA resistant strains exhibited *pncA* mutations (known or novel) with the most common G162D genotype as described previously in Hong Kong. The PCR-sequencing is highly sensitive to detect all *pncA* mutations among our PZA resistant isolates collected over 10 years (2003-2013) in Hong Kong. Another 20 confirmed PZA susceptible isolates exhibited *pncA* wild types, indicating PZA resistance is mainly associated with *pncA* mutations in our locality. This is further verified by pyrazinamidase (PZase) activity among our PZA susceptible and resistant isolates.

As *M. tuberculosis* isolates from all Hospital Authority Microbiology Laboratories are centralized in the TB reference Laboratory (PHLSB) for DST using MGIT960 system, we propose retesting all PZA-resistant isolates to provide accurate and reliable susceptibility results. Any *M. tuberculosis* clinical isolate reported as PZA resistant by the MGIT960 should be confirmed by PZase assay and *pncA* gene mutation analysis using PCR-sequencing.

C. Main Body of Report

1) Background and rationale of the research

Pyrazinamide (PZA) is a time-honored drug with unparalleled sterilizing activity in the treatment of tuberculosis (TB). In combination with rifampin, the use of pyrazinamide in the first two months has further shortened the treatment duration of drug-susceptible TB. Additionally, pyrazinamide therapy may improve the treatment outcome of pyrazinamide-susceptible multidrug-resistant TB (MDR-TB).

Phenotypic drug susceptibility testing (DST) of PZA is technically difficult. An acidic pH is required for PZA activity, but 20%-25% of clinical isolates do not grow in Lowenstein-Jensen (LJ) medium or Middlebrook 7H10/11 agar at pH 5.5. Although only 3-4% of clinical isolates are inhibited at a less acidic pH of 6.0 in liquid media (such as BACTEC 460, MGIT 960), false resistance can occur in both solid and liquid media (especially MGIT 960) as a result of inadvertent use of an excessively large inoculum. The optimal inoculum size is uncertain. At least one study has suggested that reducing the standard inoculum by 50% can reduce the PZA resistance rate from 19.5% to 3% among fully susceptible strains [Piersimoni *et al*, 2013].

2) Aims, objectives, and project indicators

We aim at evaluating whether a smaller inoculum can reduce MGIT PZA DST false resistance rate, and estimating the associated proportion with failure of growth, with the null hypothesis that the test performance with a standard inoculum size is similar to that with a reduced inoculum.

3) Study design

This study involved eligible culture isolates of *M. tuberculosis* consecutively received by Queen Mary Hospital Microbiology Laboratory serving patients in Queen Mary Hospital and Graham Hospital with *M. tuberculosis* isolated since 1 Jun 2013. Eligibility was determined by susceptibility to isoniazid and rifampicin reported by the Microbiology Division of the Public Health Laboratory Centre in Hong Kong, a WHO supranational TB Reference laboratory. This was taken as a surrogate marker of PZA susceptibility in view of the low prevalence (<5%) of

PZA resistance among non-MDR *M. tuberculosis.* If "PZA resistance" can be reduced from ~14.5% to ~4.0% by reducing the standard inoculum size, and the two-sided type I and type II errors are 5% and 20%, respectively, the minimum sample size required is 124. If 4% fails to growth, 130 independent culture isolates are required. Another 21 archived PZA resistant strains collected between 2003-2013 were also included for comparative study.

PZA susceptibility using MGIT 960 system

The MGIT 960 system (Becton Dickinson, USA) for pyrazinamide (PZA) susceptibility testing was sequentially done for each culture isolate using standard (0.5 mL) and reduced (0.25 mL) inocula to compare the proportion with PZA resistance for each inoculum size in a final concentration of PZA at 100mg/Lit. To avoid bias due to the testing sequence, order of standard or reduced inoculum was done at random.

The McNemar test was used for comparing the two methods. In case of failure to growth in either inoculum size, the culture isolate was excluded from the final analysis. The proportion with failure of growth was determined for each inoculum size.

Pyrazinamidase (PZase) activity assay

The PZase activities of the *M. tuberculosis* strains were assessed using a modified Wayne's procedure as described previously [Sigh *et al*. 2007]. Pyrazinamide (Sigma) at a final concentration of 400 mg/Lit was added to 7H10 Middlebrook agar and inoculated plates were incubated for 7 days. Ferrous ammonium sulfate was added to each plate after incubation and observed for an initial 4 h for the appearance of a pink band (positive) in the subsurface agar. Strains showing positive under the PZase activity assay were considered PZA susceptible. PZA-resistant *M. bovis* served as a negative control, and *M. tuberculosis* H37Rv served as a positive control.

Molecular detection of Pyrazinamide-resistant MTB by *pncA* PCR sequencing

The 561-nt *pncA* gene, along with surplus regions of approximately 200 nt up- and downstream of the gene, was sequenced using the pncA_F3 (AAGGCCGCGATGACACCTCT) and pncA_R4 (GTGTCGTAGAAGCGGCCGAT) primers [Jureen *et al*, 2008]. These primers were used in a standard PCR to give a template for the subsequent sequencing reactions. The pncA_F3 and

pncA_R4 primers, as well as the P3-F (ATCAGCGACTACCTGGCCGA) and P4-R (GATTGCCGACGTGTCCAGAC) primers, were used to subdivide the PCR fragment into two overlapping bidirectional sequencing reaction fragments. The sequencing reactions were performed using the BigDye Terminator cycle sequencing kit and a 3100 genetic analyzer (Applied Biosystems, Inc., Foster City, CA). Retrieved sequences were then analyzed with the (ClustalW) vector NTI Advance 9 software (InfoMax, Inc.) using the wild-type (wt) H37Rv strain's *pncA* gene (Rv2043c) as the master sequence.

BioSafety Laboratory Practice

All experiments for purified *M. tuberculosis* isolates were performed according to standard laboratory practice inside Biosafety Level III Laboratory of Dept of Microbiology, Queen Mary Hospital Compound, The University of Hong Kong.

Approval of Clinical Research Review

Approval was granted from Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB Reference Number : UW 15-087).

4) Administrative progress throughout the project period

Due to lack of expertise in *M. tuberculosis* research in Hong Kong, a Research Assistant I (Ms Kwong Tsz Ching) was employed for 10 months (March 2015 – Jan 2016) at monthly salary of $18,684.75 instead of the approved salary $19,500. A junior Research Assistant II (Mr Ng Hon Man) to assist Ms Kwong was employed for half month to finish the approved budget for personal emoluments. Staff training and reagents ordering was done in the first month of study and large scale subculture and testing for *M. tuberculosis* was initiated in the second month and screening was completed by the end of 10^{th} month. Isolates showing equivocal results or discordance were repeated and resolved by the end of 12^{th} month. All data were analyzed and compiled for report writing without delay.

5) Project results

During June 2013 to August 2014, a total of 150 *M. tuberculosis* (susceptible to isoniazid and

rifampicin) were collected from sputum of 150 patients suffering from tuberculosis (no repeated isolate from the same patient) among which 11 isolates failed in the MGIT 960 PZA susceptibility test due to under inoculums size (3 isolates for both standard and reduced inoculums; 8 isolates for reduced inoculums only). Only 139 isolates were eligible for further study and analysis. Another 21 archived PZA resistant strains collected between 2003-2013 were also included for comparative study. However, one strain was later confirmed to be PZA susceptible that 140 PZA susceptible and 20 PZA resistant strains were used for the following McNemar's test:

Table 1. 2x2 contingency table of MGIT960 PZA susceptibility results of 160 isolates for McNemar's test

Inoculum size	Reduced inoculum PZA-R	Reduced inoculum PZA-S	Row total
Standard inoculum PZA-R	20	2*	22
Standard inoculum PZA-S	2*	136	138
Column total	22	138	160

When the 4 strains (*) were repeated with the MGIT960 PZA susceptibility test, both standard and reduced inoculums were confirmed to be susceptible to PZA, negative for PZase with wild type *pncA* sequence. Using McNemar's test, there is no significant difference between standard and reduced inoculums for MGIT960 PZA susceptibility test (p = 0.6171; Odd Ratio = 1; 95% CI: 0.072 – 13.796). A total of 171 *M. tuberculosis* isolates were randomly selected for the MGIT 960 PZA susceptibility test, among which 3 isolates failed for both standard and reduced inoculums while 8 isolates failed for reduced inoculums only. The failure rate for standard and reduced inoculums was 1.8% (3/171) and 6.4% (11/171) respectively. Only 160 strains were eligible for further study.

The 21 PZA resistant archived strains collected between 2003-2013 (including one confirmed later to be PZA susceptible by MGIT960) were further characterized by MGIT960 PZA (standard and reduced inoculums) test, PZase activity and DNA sequencing to identify *pncA* mutations (Table 2).

Table 2. Characterization of PZA resistant archived strains

Strain no.	PZA susceptibility test (performed by PHLSB)	MGIT960 PZA susceptibility test (performed by HKU)		pncA mutation (Novel mutation)	PZase activity
		Standard inocula (0.5ml)	Reduced inocula (0.25ml)		
WC030	R	R	R	G162D	Negative
WC038	R	R	R	S66P	Negative
WC040	R	R	R	W68L	Negative
WC046	R	R	R	S66P	Negative
WC047	R	R	R	G Ins at nt 508	Negative
WC048	R	R	R	G162D	Negative
WC049	R	R	R	G162D	Negative
WC054	R	R	R	T Del at nt 383	Negative
WC057	R	R	R	V125D	Negative
WC059	R	R	R	G162D	Negative
WC078	R	R	R	G162D	Negative
WC106	R	R	R	G162D	Negative
WC108	R	R	R	F13S	Negative
WC120	R	R	R	11-bp del at nt 380-390	Negative
WC227	R	R	R	W68R	Negative
WC240	R	R	R	C14R	Negative
WC050	R	R	R	A Ins at nt 407	Negative
WC036	S	R	**R**	L182S	Negative
WC179	R	R	R	I90S	Negative
WC230	R	R	R	I90S	Negative
WC205	R	S	**S**	wild type	Positive
20 Rif^S INH^S susceptible strains	Not done	S	S	wild type	Positive

The most common PZA resistant mutations at pncA were G162D and I90S. Novel mutations including insertions and deletions have not been reported elsewhere. Strain WC036 (2008)

reported as PZA susceptible by TB Reference laboratory (PHLSB) was confirmed resistant to PZA by MGIT960 (both standard and reduced inoculum) with *pncA* mutations and negative for PZase activity. Strain WC205 (2013) reported as susceptible to all first line drugs but resistant to PZA by TB Reference laboratory was subsequently confirmed susceptible to PZA by MGIT960 (both standard and reduced inoculum) with wild type *pncA* and positive for PZase activity.

6) Discussion

Since PZA is considered an essential component of first-line TB therapy, it is important that laboratories should adopt a successful algorithm to provide rapid and accurate susceptibility results for PZA. The effects of inoculum concentration, volume, and homogeneity, as well as the lack of reproducibility in MGIT960 PZA susceptibility tests was reported [Chedore *et al*, 2010]. In this study, testing 160 *M. tuberculosis* isolates indicated no significant difference between standard and reduced inoculums for MGIT960 PZA susceptibility test ($p > 0.05$). The failure rate for standard and reduced inoculums was 1.8% and 6.4% respectively. Due to the potential for major errors during PZA testing with the MGIT960 assay, laboratories propose retesting all PZA-resistant isolates to provide accurate and reliable susceptibility results [Piersimoni *et al*, 2013]. Any *M. tuberculosis* clinical isolate reported as PZA resistant by the standard MGIT960 test should undergo a repeat testing using the reduced inoculum of 0.25 ml. In case of confirmation, a *pncA* gene mutation analysis and PZase assay should be performed.

Further investigation on our isolates using PCR-sequencing indicated all PZA resistant strains exhibited *pncA* mutations (known or novel) with the most common G162D genotype as described previously in Hong Kong [Chan *et al*, 2007]. The PCR-sequencing method is highly sensitive to detect all *pncA* mutations among our PZA resistant isolates collected over 10 years (2003-2013) in Hong Kong. Another 20 randomly selected PZA susceptible isolates exhibited *pncA* wild types, indicating PZA resistance is mainly associated with *pncA* mutations in our locality despite *rpsA* mutations among PZA resistant strains in a recent report [Gu *et al*, 2016]. This is further verified by PZase assay results among our PZA susceptible and resistant isolates. The 1 false susceptible and 1 false resistant isolates reported by TB reference Laboratory (PHLSB) was probably

accounted by the drug susceptibility test other than MGIT960. Since 2014, PHLSB has adopted the MGIT960 system with standard inoculums size for routine service to all Hospital Authority Hospitals in Hong Kong.

7) Conclusions

As *M. tuberculosis* isolates from all Hospital Authority Microbiology Laboratories are centralized in the TB reference Laboratory (PHLSB) for DST using MGIT960 system, we propose retesting all PZA-resistant isolates to provide accurate and reliable susceptibility results. Any *M. tuberculosis* clinical isolate reported as PZA resistant by the MGIT960 should be confirmed by PZase assay and *pncA* gene mutation analysis using PCR-sequencing.

8) References

1. Chan RC, Hui M, Chan EW, Au TK, Chin ML, Yip CK, AuYeang CK, Yeung CY, Kam KM, Yip PC, Cheng AF. Genetic and phenotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates in Hong Kong. J antimicrob Chemother. 2007 May; 59(5):866-73.
2. Chedore P, Bertucci L, Wolfe J, Sharma M, Jamieson F. 2010. Potential for erroneous results indicating resistance when using the Bactec MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. J. Clin. Microbiol. **48**:300 –301.
3. Gu Y, Yu X, Jiang G, Wang X, Ma Y, Li Y, Huang H. Pyrazinamide resistance among multidrug-resistant tuberculosis clinical isolates in a national referral center of China and its correlations with pncA, rpsA, and panD gene mutations. Diagn Microbiol Infect Dis. 2016 Mar;84(3)
4. Jureen P, Werngren J, Toro JC, Hoffner S. 2008. Pyrazinamide resistance and pncA gene mutations in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 52:1852–1854.
5. Piersimoni C, Alessandro Mustazzolu, Federico Giannoni, Stefano Bornigia, Giancarlo Gherardi, Lanfranco Fattorini. 2013 Prevention of False Resistance Results Obtained in

Testing the Susceptibility of Mycobacterium tuberculosis to Pyrazinamide with the Bactec MGIT 960 System Using a Reduced Inoculum.

6. Singh Pushpendra, Clement Wesley, G. P. S. Jadaun, Sunil Kumar Malonia, R. Das, P. Upadhyay, J. Faujdar, P. Sharma, P. Gupta, Abhay Kumar Mishra, Kalpana Singh, S. Chauhan, V. D. Sharma, U. D. Gupta, K. Venkatesan and V. M. Katoch. 2007. Comparative evaluation of Lowenstein-Jensen proportion method, BacT/ALERT 3D system, and enzymatic pyrazinamidase assay for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*. J. Clin. Microbiol. 45:76–80.