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High Resolution Melting Curve Analysis for Rapid Detection of Pyrazinamide Resistance in *Mycobacterium tuberculosis* Clinical Isolates

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Abstract

Background: Pyrazinamide (PZA) is one of the most important drugs for tuberculosis (TB) treatment, however, its susceptibility is not routinely tested. High-resolution melting (HRM) curve analysis has been widely used for many applications. In this study, HRM assay was developed and evaluated for the detection of PZA resistance in *Mycobacterium tuberculosis* clinical isolates. **Methods**: Ninety five *M. tuberculosis* clinical isolates with different susceptibility patterns to anti-TB drugs were used to evaluate this assay. Isolates were phenotypically (Bactec MGIT 960) and genotypically (HRM and *pncA* gene sequencing) analysed for PZA resistance. **Results**: Bactec MGIT 960 analysis revealed that 29 of the 95 *M. tuberculosis* isolates were PZA resistant. In comparison to the Bactec MGIT 960, HRM showed a sensitivity of 47.7% and specificity of 74.6%, and the overall agreement between the two methods was 68.4%. Based on DNA sequencing, a correlation of 0.67 (significant at p-value < 0.05) between phenotypic resistance to PZA and *pnc*A mutations was observed. PZA resistance was strongly associated with multi-drug resistant (MDR)-TB as it was shown in 79.3% of the MDR isolates included in the study. **Conclusion**: HRM is simple and useful for screening clinical *M. tuberculosis* isolates for PZA resistance, however, further modifications to improve its performance are required.

Keywords

Pyrazinamide, Tuberculosis, High Resolution Melting Curve Analysis, Drug Resistance, *pnc*A Gene

1. Background

Pyrazinamide (PZA) is one of the most important drugs for tuberculosis (TB) treatment. It has a unique sterilizing effect on killing semi-dormant bacilli and is also very effective against multi-drug (MDR)-TB [1] [2]. Pyrozinoic acid (POA) is the active form of the drug and is metabolized by pyrazinamidase (PZase) enzyme which is encoded by the *pnc*A gene [3]. Although number of studies suggest that PZA resistance is not caused by a single mechanism, mutations in the *pnc*A gene are considered as the main mechanism of PZA resistance in *M. tuberculosis* [4]. The lack of reliable culture-based methods which require an acidic condition and high cost of the molecular techniques makes the testing of PZA resistance generally very difficult to perform [5]. It is very important to develop rapid and accurate drug susceptibility testing (DST) to prevent the spread of multi-drug resistant (MDR)-TB as well as extremely drug resistant (XDR)-TB.

Several molecular techniques have been described for the detection of anti-TB drug resistance-associated mutations, including the line probe assays GenoType MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany), GenoType MTBDRsl (Hain Lifescience GmbH, Nehren, Germany), INNO-LiPA Rif.TB (Innogenetics, Ghent, Belgium), and Xpert MTB/RIF (Cepheid, Sunnyvale, CA) [6] [7]. However, these are probe based assays and only suitable for mutations that are located in hotspot regions of specific genes. Mutations in the *pnc*A are dispersed throughout the gene and its upstream promoter; this makes the development of probe-based methods challenging [8]. Direct sequencing of *pnc*A amplicons remains the best genotypic strategy, however this is costly.

High-resolution melting (HRM) curve analysis is a simple technique and has been widely used for many applications. PCR amplicons are heated and fluorescence loss is monitored in real-time. Variations in sequences are detected by difference in melting point (T*m*) compared to reference DNA; it does not require the use specific probes [9] [10]. In the TB field, HRM has been used for detecting rifampin (RIF), isoniazid (INH), streptomycin (STR), and fluoroquinolone resistant *M. tuberculosis* [11]. In this study, we describe an HRM technique to detect *pnc*A mutations in *M. tuberculosis* clinical isolates and compare its results to the phenotypic PZA susceptibility testing (The Bactec MGIT 960). Isolates that had discordant results were sent for sequencing. This method uses the Roche LightCycler 480® and involves simultaneous amplification of three overlapping fragments.

2. Methods

The study was conducted at the NHLS-TB Laboratory Tshwane Academic Division (Diagnostic division of the Department of Medical Microbiology, University of Pretoria from July 2013 to October 2013. Ethics approval was obtained (number 57/2012) prior to commencement of the study. The study isolates (95) were a mixture of MDR-TB and non-MDR-TB collected from the National Health Laboratory Service (NHLS) TB Laboratory. All isolates were presumptively identified as *M. tuberculosis* complex using Ziehl-Neelsen (ZN) stain and tested for susceptibility using the GenoType MTBDR *plus*

line probe assay (HainLifescience GmbH, Germany). Isolates were then categorized as either MDRs or Non-MDRs (Mono-RIF resistance, Mono-INH resistance or fully sensitive) [7].

Purity of all isolates by ZN staining was confirmed before susceptibility testing was performed. To exclude bacterial and fungal contamination, another amount of a well-mixed suspension was inoculated using sterile glass pipette on chocolate agar (produced in-house, NHLS Laboratory, Tshwane Academic Division) and incubated aerobically at 37˚C. All plates were inspected for bacterial growth every 24 hrs for two days.

Isolates were sub-cultured onto 7H10 agar medium (Becton Dickinson, Sparks, MD, USA) and incubated for three weeks aerobically at 37˚C until typical *M. tuberculosis* colonies appeared (dry, wrinkled, warty, with colourless rough surface). Colonies were picked and sub-cultured into MGIT tubes (Becton Dickinson, Sparks, MD, USA) according to the manufacturer's procedure.

Each isolate was tested for PZA susceptibility when the Bactec MGIT 960 indicated positive culture growth. The day the Bactec MGIT 960 (Becton Dickinson, Sparks, MD, USA) gives a positive signal is considered as day zero. Once positive, tubes were removed from the Bactec MGIT 960 (Becton Dickinson, Sparks, MD, USA) they were transferred into an incubator 37˚C ± 1˚C until susceptibility testing was performed (within five days). Undiluted inoculum was used if the isolate was processed on days one or two. On days three, four or five, the growth culture was diluted 1:5 using normal saline (0.9%) (SABAX Pour Saline, Adcock Ingram, South Africa) prior to susceptibility testing. PZA susceptibility testing was performed according to the manufacturer's procedure (Becton Dickinson, Sparks, MD, USA). Susceptibility results were available within four to 21 days. Once the test was completed, a report was generated and printed. Each isolate was categorized as "S" (susceptible) or "R" (resistant).

2.1. DNA Extraction of *M. tuberculosis* Isolates

Genomic DNA was extracted from the *M. tuberculosis* isolates using QIAquick PCR Purification Kit (Whitehead Scientific, Brackenfell, South Africa) according to the manufacture's procedure. Extracted DNA was quantified using specific spectrophotometry (NanoDrop; ND-1000; USA), and adjusted to a concentration of five to 30 ng/µl using elution buffer (BioMérieux, France), this range is required by HRM for optimal performance. The extracted DNA was then transferred into a sterile 1.5 ml tube (BioMérieux, France) and used directly for HRM analysis or stored at −80˚C.

2.2. HRM Optimization

The LightCycler® 480 HRM master mix contains all the ingredients required. The two most important contents which needed to be optimized carefully before commencing with the testing process were $MgCl_2$ and primers.

2.2.1. $MgCl_2$

Because specific amplification is essential for HRM analysis, determination of $MgCl_2$

optimum concentration for each new primer pair is very important. A separate 25 mM $MgCl_2$ stock solution was supplied with the master mix. This is to allow the easy optimization of Mg^{2+} concentration. Optimization was achieved by running a positive sample with serial dilutions of $MgCl_2$ (1.0, 1.5, 2.0, 2.5, 3.0 3.5 mM) and analysis of the PCR products by agarose gel electrophoresis.

2.2.2. Primers

Primers previously described by Scorpio *et al*. were synthesized by Inqaba Biotechnical Industries Pretoria, South Africa to the *M. tuberculosis pnc*A sequence (GenBank accession number U59967) [12] (**Table 1**). Prior to the experiment and to ensure specific amplification for HRM analysis, the optimal primer concentration (the lowest concentration that still results in a high rate of amplicon yield with a low cut off point (Cp) and adequate fluorescence dynamics for a given target concentration) was determined. This was achieved by testing different concentrations of each primer (0.1, 0.2 and 0.3 μM).

2.3. Real-Time PCR and HRM Conditions

HRM curve analysis including the amplification step was performed using the LightCycler® 480 High Resolution Melting Master Kit and the LightCycler® 480 Instrument II (Roche, Germany). In order to detect all possible mutations in the *pnc*A gene (561 bp) regardless of their locations, and to achieve the recommended size of the gene for HRM analysis (less than 300 bp), the target gene was amplified using three sets of primers to produce three fragments of the target gene [12].

The reaction mixture was prepared according to the manufacturer's instructions (LightCycler 480® Real-Time PCR manual, 2009). HRM master mix consisted of 2× a concentration hot-start reaction mix that contained FastStartTaq DNA polymerase, reaction buffer, dNTP mix (with dUTP) and HRM Dye. PCR reaction composition is shown in **Table 2**. Inclusion of HRM dye enabled detection of double-stranded DNA by fluorescence, monitoring formation of amplicon during PCR cycling and melting curve analysis.

These components were added into a 1.5 ml reaction tube on ice and mixed carefully by pipetting up and down. The amount in the volume column was multiplied by the

Table 1. Primers sequences of used for the amplification of *pnc*A gene.

Primers		Sequences 5'……..3'
Set 1	P1	F: GTCGGTCATGTTCGCGATCG
	P2	R: TCGGCCAGGTAGTCGCTGAT
Set 2	P3	F: ATCAGCGACTACCTGGCCGA
	P4	R: GATTGCCGACGTGTCCAGAC
Set 3	P5	F: CCACCGATCATTGTGTGCGC
	P6	R: GCTTTGCGGCGAGCGCTCCA

Table 2. Reaction mixture used in HRM analysis for the detection of *pnc*A gene mutation in *M. tuberculosis*.

Reagents	Volume (µl)×1	Final Concentration
Master Mix, 2× conc.	10 µl	1× conc.
$Mgcl_2$, 25 mM	1.6 µl	2 mM
Primer mix, 20 × conc. {4 µM}	1.0 µl	0.2 µM
Template DNA	5.0 µl	5 - 30 ng
Water, PCR-grade	2.4 µl	-
Total volume	20 µl	-

number of reactions to be run. Fifteen µl of the PCR mixture was transferred into each well of the LightCycler® 480 Instrument multi-well plate (Roche, Germany) then 5 µl of the DNA template was added, mixed by pipetting up and down. The LightCycler® 480 foil was used to seal the multi-well plate before loading it into the LightCycler® 480 (Roche, Germany) (Table 3).

A non-template control containing sterile distilled water was included in the experiment. *M. tuberculosis* wild type strain American Type Culture Collection (ATCC H37) as well as a *M. bovis* strain served as negative and positive controls for the *pnc*A mutation respectively. Collected data were analysed following the final step using Light Cycler 480 software version 1.5. Temperature plots were generated by converting the wild type melting profile to a horizontal line and normalizing the melting profiles of the examined isolates against the *M. tuberculosis* wild-type profile. *M. tuberculosis* isolates with mutations in the *pnc*A sequence were distinguished from the wild type based on the difference in melting temperature observed. At the end of the analysis, the software either reported the isolate as a wild type (PZA susceptible) or mutant (PZA resistant).

2.4. DNA Sequencing of the *pnc*A Gene of Discordant *M. tuberculosis* Isolates

The entire *pnc*A gene was sequenced using two primers, P1 and P6, which flank the entire *pnc*A gene and its upstream promoter (700-bp). Purified DNA (10 µl) from each isolate was sent for DNA sequencing which was carried out by a commercial sequencing centre using standard Sanger sequencing (InqabaBiotec, SA). Retrieved sequences were compared with the wild type *pnc*A sequence from *M. tuberculosis* H37Rv for the detection of mutations associated with PZA resistance. Sequence alignment and analysis was performed using special software (CLC Genomics workbench, Denmark). Isolates were either reported as wild type (PZA susceptible) or mutant (PZA resistant). These results were compared to the HRM results, as well as the phenotypic drug susceptibility results obtained from the Bactec MGIT 960 to assess the HRM against a molecular gold standard as well.

2.5. Statistical Analysis

Statistical analysis was performed using the Bactec MGIT 960 as a phenotypic gold

Table 3. PCR amplification and HRM conditions.

Steps in the PCR cycle	Temperature	Time	No. of cycles
Initial denaturation (FastStartTaq DNA Polymerase)	95˚C	10:00 min	1
Denaturation	95˚C	00:10 sec	45
Annealing	63˚C	15:00 min	
Extension	72˚C	00:16 sec	
	For HRM		
Second hold	95˚C	1:00 min	1
	40˚C	00:01 min	
	65˚C	00:01 sec	
Second temperature increase	95˚C	Fluorescent detection (25 acquisition per 1˚C)	
Cooling down	40˚C	00:10 sec	

standard method and DNA sequencing as a molecular gold standard method. Results of HRM and MGIT or sequencing susceptibility testing were entered into 2 × 2 tables to calculate the sensitivity (ability to detect true resistance) and specificity (ability to detect true susceptibility) of HRM against MGIT as well as the positive predictive values (PPV) and the negative predictive values (NPV).

3. Results

A total of 120 *M. tuberculosis* isolates were collected from the NHLS-TB Laboratory Tshwane Academic Division (Diagnostic division of the Department of Medical Microbiology, University of Pretoria. Seven isolates were excluded due to duplication. Additional 18 isolates had no HRM results and were excluded from analysis. Susceptibility of the 95 *M. tuberculosis* isolates included in the final analysis to the first-line anti-TB drugs are shown in Figure 1. Isolates were categorized as MDR-TB (Resistant to both RIF and INH) and Non-MDR-TB (susceptible to at least RIF or/and INH).

3.1. The BACTEC MGIT 960

According to the Bactec MGIT 960, 30.5% (29/95) of the isolates were phenotypically resistant to PZA. Of these 79.3% (23/29) were MDR and 20.7% (6/29) were non-MDR. Prazinamide susceptible isolates were detected in 69.4% (66/95) of the isolates, in which 25.8% (17/66) were MDR and 74.2% (49/66) were non-MDR (Table 4). Of the 34 isolates that were phenotypically susceptible to other drugs, 33 were determined to be susceptible to PZA. However, one isolate, which was phenotypically susceptible to other drugs, was PZA-resistant, suggesting PZA mono-resistance (Table 4). The average time of reporting PZA susceptibility results was 8.5 days and 86.3% of isolates were reported in four to 12 days.

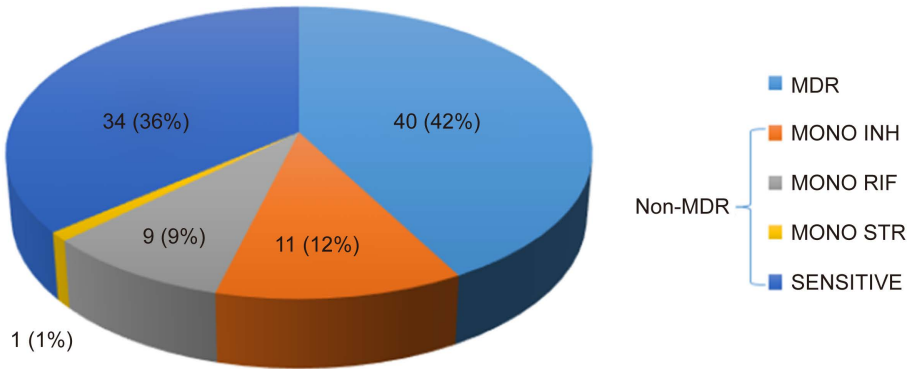


Figure 1. First-line susceptibility profile of 95 *M. tuberculosis* isolates included in the study. MDR = Multi-drug resistant (Resistant to at least RIF and INH); Mono INH = Resistant to INH only; Mono RIF = Resistant to RIF only; Mono STR = Resistant to STR only; Sensitive = Susceptible to both RIF and INH.

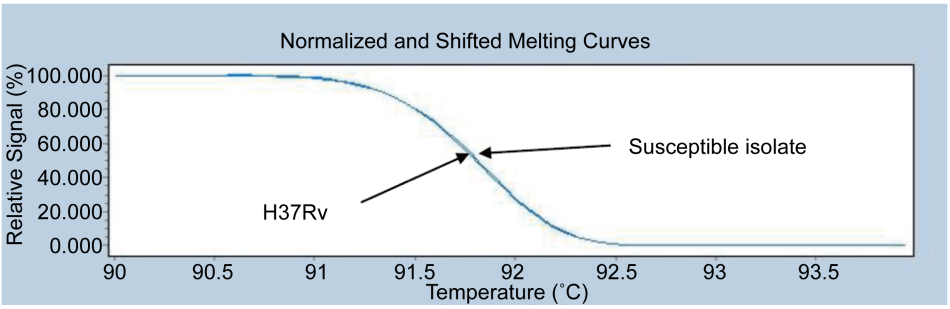
Table 4. PZA susceptibility results by the BACTEC MGIT 960.

M. tuberculosis Isolates	BACTEC MGIT 960	
	R	S
MDR-TB (*n* = 40)	23	17
Non-MDR-TB (*n* = 55)	6	49
Mono Rif (***n* = 9**)	1	8
Mono INH (***n* = 11**)	3	8
Mono STR (***n* = 1**)	1	0
Sensitive (***n* = 34**)	1	33

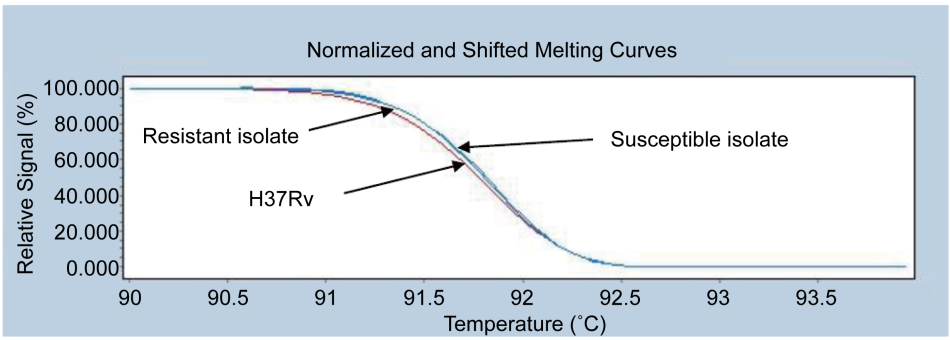
MGIT = Mycobacterial Growth Indicator Tube; MDR–TB = Multi-drug resistant tuberculosis; R = Resistant; S = Susceptible; Mono INH = Resistant to INH only; Mono RIF = Resistant to RIF only; Mono STR = Resistant to STR only; Sensitive = Susceptible to both RIF and INH.

3.2. HRM Analysis

High Resolution Melting curve analysis of the *pnc*A gene was performed in DNA samples isolated from 95 *M. tuberculosis* isolates using three overlapping fragments. Representative normalized melting curves from HRM analysis are shown in **Figure 2(a)** and **Figure 2(b)**. In the HRM graphs, each line indicates the melt curve profile for an individual sample. The normalized graph shows melting temperature shifts versus wild-type amplicon indicating lower or higher melting temperature. Based on the differences in the shape of the melting curves in any of the three overlapping fragments, *M. tuberculosis* isolates with mutations could easily be differentiated from the susceptible (wild-type) ones. Wild types are represented by blue lines, whereas mutants are represented by red lines. In each batch, DNA isolated from the susceptible control strain (H37Rv) was included as a reference and shown to be wild type at all times (blue line). Based on HRM analysis, 77.9% (74/95) of the isolates were susceptible to PZA while 22.1% (21/95) were resistant.



(a)



(b)

Figure 2. Normalized melting curves from HRM analysis of the *pnc*A gene. (a): Amplicons amplified from susceptible isolate versus wild-type reference strain (H37Rv); (b): Amplicons amplified from susceptible and resistant isolates versus wild-type reference strain (H37Rv).

3.3. Comparison of PZA Susceptibility Testing Results for the BACTEC MGIT 960 and HRM Analysis

Of the 95 isolates included in this study, 55 isolates (57.9%) and 10 isolates (10.5%) were have shown resistance and susceptibility by both methods, respectively. Discordant results were observed in 30 isolates (31.6%). When the Bactec MGIT 960 method was considered as the gold standard, HRM showed a sensitivity (ability to detect true resistance) of 47.7% and specificity (ability to detect true susceptibility) of 74.6%. The accuracy, the positive and negative predictive value of HRM were 69.5%, 34.5% and 83.3%, respectively. The overall agreement between the two methods was 68.4% (Table 5).

3.4. DNA Sequencing of *pnc*A Gene

PCR products were obtained from 48 isolates (50.5% of the total sample size) and sequenced. These included all isolates that showed resistance to PZA by MGIT 960 (30), all isolates that showed resistance by HRM (11) and seven isolates that were randomly selected from those which were susceptible by both methods i.e. MGIT 960 and HRM (Table 6).

Different types of mutations in the *pnc*A-encoding region were identified in 27 isolates (56.3%), including nucleotide substitutions, insertions and deletions. No multiple-site mutation was detected (Table 6). Substitution of a single nucleotide was the most

Table 5. Comparison of PZA susceptibility testing results for the BACTEC MGIT 960 and HRM curve analysis.

Test	MGIT 960	HRM	Number of isolates with results
Results for indicated test	S	S	**55**
	R	R	**10**
	S	R	**11**
	R	S	**19**

Performance parameters:
Sensitivity = 47.7%
Specificity = 74.6%
Accuracy = 69.5%
PPV = 34.5%
NPV = 83.3%

MGIT = Mycobacterial Growth Indicator Tube; HRM: High Resolution Melting; S = Susceptible, R = Resistant; PPV: Positive predictive value; NPV: Negative predictive value.

Table 6. Drug susceptibility patterns and DNA sequencing of *pncA* gene of 48 *M. tuberculosis* isolates.

Isolate Number	Resistance Pattern	PZA susceptibility testing		DNA Sequencing of *pncA* gene				
		MGIT	HRM (F)	Type of change	Codon number[¤]	Nucleotide change		Amino acid change
						From	To	
1	RIF, INH,STR, EMB, ETH, KAN, OFX	R	S	Insertion	173	-	C	Glu173Frameshift **
2	INH	S	R (F3)	No change	-	-	-	-
3	RIF, INH	R	S	Substitution	59	A	G	Ser59Pro **
4	RIF, INH, OFX	R	S	Substitution	14	A	G	Cys14Arg *
5	IHN, STR	R	S	Substitution	51	G	C	His51Asp **
6	INH, STR, EMB	R	R (F1)	No change	-	-	-	-
7	RIF, INH	R	R (F1)	Substitution	139	A	C	Va139Gly *
8	INH,STR, ETH	S	S	No change	-	-	-	-
9	RIF, INH	R	S	Substitution	14	G	C	Cys14Trp **
10	RIF, INH	S	R (F1)	No change	-	-	-	-
11	RIF, INH	R	R (F2, F3)	Substitution	14	G	C	Cys14Trp **
12	INH	R	R (F3)	No change	-	-	-	-
13	RIF, INH	S	S	No change	-	-	-	-
14	RIF, INH	R	S	Substitution	134	G	T	Ala134Asp **
15	RIF, INH	S	R (F3)	Substitution	139	A	C	Val139Gly *
16	RIF, INH, ETH	R	R (F3)	Substitution	12	T	C	Asp12Gly *
17	INH	S	R (F3)	Substitution	34	A	C	Tyr34Asp **
18	RIF, INH, EMB, OFX	R	R (F1, F2, F3)	No change	-	-	-	-
19	RIF, INH	R	R (F1, F2, F3)	No change	-	-	-	-

Continued

20	RIF, INH	R	R (F1, F2, F3)	Deletion	21	A	-	Val21Frameshift **
21	RIF, INH OFX	S	S	Substitution	10	G	A	Ala10Val *
22	RIF, INH, EMB	R	R (F1)	Substitution	14	A	G	Cys14Arg *
23	RIF, INH	S	S	Substitution	14	G	C	Cys14Trp **
24	STR	R	S	No change	-	-	-	-
25	RIF, INH	R	S	No change	-	-	-	-
26	RIF, INH	S	S	No change	-	-	-	-
27	RIF	R	S	No change	-	-	-	-
28	RIF, INH, STR, EMB	R	S	Substitution	135	T	G	Thr135Pro *
29	RIF, INH	S	R (F3)	Substitution	139	A	C	Val139Gly *
30	RIF	S	R (F1)	Substitution	172	A	G	Leu172Pro *
31	RIF, INH	R	R (F2)	Substitution	59	A	G	Ser59Pro **
32	RIF, INH	R	S	Substitution	135	T	G	Thr135Pro *
33	RIF, INH	R	S	Substitution	135	T	G	Thr135Pro *
34	RIF, INH, EMB	R	S	Substitution	135	T	G	Thr135Pro *
35	RIF, INH, STR	R	S	Substitution	135	T	G	Thr135Pro *
36	RIF, INH	R	S	No change	-	-	-	-
37	RIF, INH	R	S	Insertion	12	-	C	Asp12Frameshift **
38	Susceptible	S	R (F1, F3)	Substitution	122	G	A	Gln122Stop **
39	RIF, INH	R	S	Substitution	12	T	C	Asp12Gly *
40	RIF, INH	R	S	No change	-	-	-	-
41	Susceptible	S	S	No change	-	-	-	-
42	Susceptible	S	R (F3)	No change	-	-	-	-
43	Susceptible	S	S	No change	-	-	-	-
44	Susceptible	S	S	No change	-	-	-	-
45	Susceptible	S	S	No change	-	-	-	-
46	Susceptible	R	S	Substitution	57	G	C	His57Asp **
47	Susceptible	S	R (F1, F2, F3)	No change	-	-	-	-
48	Susceptible	S	R (F2, F3)	No change	-	-	-	-
H37Rv	Susceptible	S	S	No change	-	-	-	-

PZA = Pyrazinamide; MGIT = Mycobacterial Growth Indicator Tube; HRM: High Resolution Melting; (F): Fragment showing variation by HRM; RIF = Rifampicin; INH = Isoniazid; STR = Streptomycin; EMB = Ethambutol; ETH = Ethionamide; KAN = Kanamycin; OFX = Ofloxacin; S = Susceptible; R = Resistant; A = Adenine; T = Thymine; C = Cytosine; G = Guanine; H37Rv = Control strain (Wild-type); (¤) = Number of codon position was counted from the start codon (ATG) of the *pnc*A; (*) = High confidence mutations (mutations associated with *in-vitro* documented resistance reported by at least 10 publications); (**) = New mutations (reported for the first time).

common mutation 88.9% (24/27), followed by insertion 7.4% (2/27) and deletion 3.7% (1/27). No mutation was detected in 21 (43.7%) isolates, of these, four (19%) were resistant by both methods, five (23.8%) were resistant by MGIT 960 only, five (23.8%) were resistant by HRM only and seven (33.4%) were susceptible by both methods (Table 6).

As indicated in Table 6, the mutation rate among the phenotypically resistant isolates that were reported by MGIT 960 was 65.5% (19/29), whereas mutations were only detected in 45.5% (5/11) isolates that were initially reported as resistant by HRM. Additionally, mutations were shown in 60% (6/10) of the isolates that were reported resistant by both methods. No mutation was identified in the wild-type (H37Rv) strain. However, two isolates, despite being susceptible by both MGIT and HRM, showed mutations in the *pnc*A gene. High confidence mutations, *i.e.* mutations associated with in-vitro documented resistance reported by at least 10 publications were found in 51.8% (14/27) of isolates. The rest, 48.2% (13/27), were new mutations which have not been reported in previous studies.

The most frequently mutated sites were at codon 135, which showed a substitution from thymine to guanine at nucleotide 403, this resulted in a switch at translation from threonine to proline in five isolates (18.5%). Another common site was codon 139 which showed a substitution from adenine to cytosine at nucleotide 416, and resulted in a switch at translation from valine to glycine in three isolates (11.1%). Additional three isolates (11.1%) showed new mutations in codon 14, switching translation from cysteine to tryptophan. These three mutation sites accounted for more than one third of all the mutations identified (Table 6).

3.5. Comparison of HRM Analysis and the BACTEC MGIT 960 with DNA Sequencing

Isolates with discordant results between HRM analysis and the Bactec MGIT PZA susceptibility were compared with DNA sequencing of the *pnc*A gene (Table 7). HRM analysis was concordant with DNA sequencing for 47.9% (23/48) of the isolates. Among the 25 discordant isolates, 16 isolates had mutations in the *pnc*A gene while HRM detected no variation; and 9 had no mutations in the *pnc*A while HRM detected variation. DNA Sequencing of *pnc*A was concordant with the Bactec MGIT 960 PZA susceptibility testing for 66.7% (32/48) of the isolates. Among the 16 discordant isolates, nine were PZA resistant while sequencing detected no mutation, and seven were PZA susceptible while sequencing showed mutations. Overall, there were fewer discrepancies with MGIT than with HRM.

4. Discussion

Although PZA forms an integral part of both first line and MDR TB management, routine susceptibility testing of this drug is not performed due to various challenges present in the currently available techniques. This study evaluated the rapid technique of HRM for the detection of PZA resistance against the phenotypic gold standard (Bactec

Table 7. Comparison of HRM analysis and The BACTEC MGIT 960 with DNA sequencing.

DNA Sequencing	HRM PZA Susceptibility (n = 48)		DNA Sequencing	MGIT PZA Susceptibility (n = 48)	
	Susceptible	Resistant		Susceptible	Resistant
Mutation	16	11	Mutation	7	20
No mutation	12	9	No mutation	12	9
	Performance parameters: Sensitivity = 40.74% Specificity = 57.14% Accuracy = 47.9% PPV = 55.0% NPV = 42.86%			**Performance parameters:** Sensitivity = 68.97% Specificity = 63.16% Accuracy = 66.7% PPV = 74.07% NPV = 57.14%	

MGIT = Mycobacterial Growth Indicator Tube; **HRM**: High Resolution Melting; **PZA**: Pyrazinamide**; PPV**: Positive predictive value; **NPV**: Negative predictive value.

MGIT 960).

In this study, the Bactec MGIT 960 results showed a prevalence of PZA resistance of 57.5% (23/40) among the MDR-TB isolates tested, while the prevalence among non-MDR isolates was 10.9% (6/55). High rates of PZA resistance among MDR-TB have been observed in previous studies, ranging from 49% in Thailand [13], to 50% in Central Africa [14], 52% in South Africa [15], 53% in Japan [16] and 55% in Taiwan [17]. Studies from Pakistan [18], South Korea Kim *et al*., 2012 [19] and India [20] have shown higher rates of resistance, 77%, 85% and 85% respectively. These high rates of PZA resistance in TB endemic countries may be attributed to the widespread use of PZA in re-treatment regimens in these areas, particularly when used without relying on susceptibility testing results. The high occurrence of false resistance by phenotypic susceptibility testing could also cause the overestimation of PZA resistance among MDR-TB [21].

In 2013, Nagai *et al.* developed and evaluated HRM curve analysis for mutation detection in four major anti-TB drugs, namely RIF, INH, EMB, and STR. Their HRM assay was successful in detecting mutations in genes associated with resistance (*rpo*B, *kat*G, *inh*A, *amb*B, *rps*L and *rrs*) and results were completely consistent with those of DNA sequencing. Sensitivity and specificity of HRM were 100% and 100% for RIF, 88.8% and 100% for INH, 100% and 100% for EMB, and 100% and 93.7% for STR, respectively [22].

Comparable results were also achieved by another two recent studies when HRM was used for the detection of PZA resistance. Hong *et al.* reported a sensitivity and specificity of 85.5% and 98.5%, respectively. Whereas Pholwat *et al.* showed concordance of 84% between HRM and phenotypic PZA susceptibility testing [23] [24]. The agreement between the phenotypic Bactec MGIT 960 and HRM for PZA susceptibility in this study was only 68.4%. Sensitivity and specificity of HRM were 47.7% and 74.6%, respectively. These values were much lower than previously reported [24] [25] [26]. Discrepancies between the two methods detected on 19 PZA-resistant isolates that were reported wild-type by HRM, and on 10 PZA-susceptible isolates that showed mutations by HRM. Among these 19 isolates, sequencing detected mutations in 14 of them sug-

gesting that HRM was not able to detect these mutants which were also phenotypically resistant. The majority variants were SNPs with five of the 19 occurring at codon 135.

Conversely, five of the ten susceptible isolates which were reported as variants by HRM showed no mutation on sequencing. Although this would suggest that these were false on HRM, it is also possible that the HRM result is true since HRM is PCR based and has a greater likelihood of detecting mixed populations which may not be detected by Sanger sequencing, and these sub-populations may not be large enough to show resistance phenotypically. We did not perform next generation sequencing which may have provided an answer to this uncertainty.

Recent studies have indicated that the new "gold standard" for PZA resistance determination should be sequencing. When considering the discordant isolates only and comparing HRM and MGIT 960 against this "new" gold standard, the accuracy for HRM was 47.9% while that of the Bactec MGIT 960 was slightly better at 66.7% (**Table 7**).

Phenotypic resistance to PZA has been correlated with mutations in the *pnc*A gene in several previous studies. Some of these studies have reported this correlation to be inconsistent, ranging between 41% and 80% [25]-[31], whereas other studies showed higher correlation ranging from 91% - 97% [15] [17] [32] [33] [34]. In the current study, based on sequencing results, a correlation of 0.67 (significant at p-value < 0.05) between phenotypic resistance to PZA and *pnc*A mutation was observed, this is comparable to majority of the previous studies, more especially to the study that was conducted in South Africa by Bishop *et al.*, in which a correlation of 67% was also reported [35].

The mutations detected in our study were found to be scattered along the entire *pnc*A gene with no major hot spots identified. However, mutations at codon 135 were found in five isolates, whereas mutations at codons 14 and 139 were shown by three isolates each. These observations of scattered mutations are supported by similar findings in previous studies [27] [36]. The high diversity of *pnc*A mutations limits its inclusion in the current molecular techniques such as GenoType MTBDR*plus* (Hain Lifescience) [37].

Absence of mutations in the *pnc*A gene or its upper promoter in 26.7% of the PZA-resistant isolates we tested correlated with the study that was done by Sreevatsan *et al.*, in which they reported no *pnc*A mutations in 28% of PZA-resistant *M. tuberculosis* strains tested [15]. These cases could also be explained by another mechanism of resistance to PZA. Recently, *rps*A gene has been shown to play a role in PZA resistance and has been recommended to be used as an additional target for the molecular detection of PZA susceptibility [9] [38], however in another study, none of the PZA-resistant strains harboured mutations in the *rps*A gene [39]. Based on these variations, the association between PZA resistance and *rps*A mutations remains un-established and requires further investigations in the future.

The low sensitivity and specificity of HRM in this study could be due to the use of three sets of primers as compared to five or seven as used in previous studies [40] [41].

Unlike other studies, the current HRM was developed and evaluated using the Roche LightCycler® 480. So far, only one study has utilized the Roche LightCycler 480® for PZA susceptibility testing and used the Bactec MGIT 960 as a reference method [42]. They have shown a sensitivity and specificity of 85.5% and 98.5%, respectively and concluded that HRM is a rapid and accurate test for the detection of PZA resistance and can be used as a screening method [26]. However, recently, HRM results generated from the Rotor-gene system for the detection of PZA resistance have been found to be easier to interpret than other platforms [25].

Another explanation for the low correlation between PZA resistance and *pnc*A mutations in this study is the possibility of phenotypic false resistance. This observation has been proven elsewhere [25]. PZA phenotypic susceptibility testing by the Bactec MGIT 960 is normally carried out at pH of 5.9, resistance could be falsely reported as a result of the ammonia produced during mycobacterial growth; ammonia elevates the pH of the media thus inactivating the drug [26]. According to the Henderson-Hasselbalch equation, *M. tuberculosis* should be tested for PZA susceptibility at a concentration of at least 156 μg/ml [10], rather than the current cut-off used in the Bactec MGIT 960 method (100 μg/ml). PZA resistance could also be caused by the action of efflux pumps which has been shown to play a role in mycobacterial resistance [43]. Detection of *pnc*A mutations in the susceptible isolates suggests that these mutations might not be fully expressed to show phenotypic PZA resistance.

5. Conclusions

In this study, apart from simplicity and speed, the current HRM genotypic method to determine PZA susceptibility using the gene scanning software of the Roche LightCycler 480 was shown to be moderately specific with low sensitivity. Further modifications to improve its performance are required. Pyrazinamide resistance was common among MDR-TB and the current study results showed low association of the phenotypic PZA resistance with mutations in the *pnc*A gene. This warrants further studies to determine potential alternate genetic mechanisms of resistance to PZA in our context and re-evaluation of the MGIT 960 for detection of phenotypic resistance with an alternate phenotypic method such as the Wayne's test.

This study provides an important baseline for more extensive evaluation studies to improve and validate the use of HRM in determining PZA susceptibility. In the future, using more than three fragments (six or seven) should be considered as this could increase the sensitivity and specificity of HRM in detecting *pnc*A mutations. However, this might also increase the cost of the test. Additionally, combining such data to results from the biochemical analysis of PZase enzyme (Wayne's test) would add to its value and ease the interpretation of the final results.

Conflict of Interest

The authors declare that they have no financial or non-financial competing interests.

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