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Pyrazinamide susceptibility testing of Mycobacterium tuberculosis by high resolution melt analysis

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Abstract

Pyrazinamide (PZA) plays the important role in shortening the tuberculosis treatment period and in treating MDR-TB. Phenotypic PZA susceptibility methods are limited because they require specialized acidified media, which increases costs and complexity. In this study we developed a genotypic high resolution melt (HRM) analysis technique to detect *pncA* mutations associated with PZA resistant *M. tuberculosis*. Seven overlapping primer pairs were designed to cover the entire *pncA* gene and upstream regions. Each gene segment was individually amplified by real-time PCR followed by HRM analysis. The assay was evaluated on 98 clinical *M. tuberculosis* isolates (41 PZA susceptible by MGIT method, 55 PZA resistant, 2 undetermined). HRM was 94% concordant to full-length sequencing results, with most discrepancies attributable to mixed populations per HRM or transversions. Sequencing and HRM yielded 82% and 84% concordance, respectively, to phenotypic PZA susceptibilities by MGIT, with most discrepancies attributable to isolates with wild-type *pncA* but phenotypic PZA resistance. This HRM technique is a simple and high-throughput method for screening clinical *M. tuberculosis* samples for PZA resistance.

Keywords

Pyrazinamide; High resolution melt; genotypic; MDR-TB

INTRODUCTION

Pyrazinamide (PZA) is a first-line drug for the treatment of tuberculosis. Its use allows shortening of the treatment period from 9 to 6 months and it is also widely included in regimens for MDR-Tb.[1–4] The importance of PZA susceptibility testing has increased due to

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ETHICAL APPROVAL

All work was approved by the University of Virginia Institutional Biosafety Committee and Human Investigation Committees and was conducted in compliance with the Declaration of Helsinki.

COMPETING INTERESTS: None.

the recognition of PZA monoresistant strains of *M. tuberculosis* and the need for improved MDR-TB regimens, since some new TB drugs such as bedaquiline appear to benefit from PZA.[5, 6] Unfortunately conventional susceptibility testing for PZA is limited by the requirement for acidic media, which inhibits the growth of *M. tuberculosis*.[7] The radiometric BACTEC 460 system is no longer available and the newer non-radiometric BACTEC MGIT 960 system requires 8 to 12 days, has potential for cross contamination, and false resistance has been reported.[8–10]

PZA is a prodrug that requires activation to its active metabolite pyrazinoic acid by pyrazinamidase, which is encoded by the *pncA* gene.[11] A correlation of approximately 85% has been observed between phenotypic PZA resistance and *pncA* mutation.[12–14] A number of molecular methods have been developed for the detection of other TB drug resistance-associated mutations including the line probe assays MTBDRplus, MTBDRsl, INNO-LiPA Rif.TB, and Xpert MTB/RIF.[15–17] These probe based assay are suitable for commonly known mutations in hotspot regions of specific genes. *pncA* mutations associated with PZA resistance, however, appear to be dispersed throughout the 561 base pair gene and an upstream promoter-containing region, several hundred base pairs overall, which makes the development of probe-based methods complex.[12–14] A gel based PCR–single strand conformation polymorphism method has been used as has a temperature-mediated heteroduplex method.[14, 18] There are also line probe assays which use multiple (e.g., 49) probes for reverse hybridization after nested PCR.[19, 20] Such methods are laborious and prone to amplicon contamination. Closed systems are now feasible, including melt-based assays with sloppy molecular beacons or lights-on/lights-off probes[21–23] yet such strategies for *pncA* would still require complex design and utilization of dozens of probes. Direct sequencing of *pncA* amplicon is in our view the best genotypic strategy, however this requires a costly apparatus.

High-resolution melt (HRM) curve analysis is a simple technique. After endpoint PCR with a fluorescent dye, PCR amplicons are heated and fluorescence loss is monitored in real-time. Sequence variants are detected by differing melt profiles from reference DNA without the need for specific probes. HRM has been widely utilized for a variety of applications.[24–26] In the tuberculosis arena, HRM has been used for detecting rifampin, isoniazid, streptomycin, and fluoroquinolone resistant *M. tuberculosis*.[27–31] In this study, we describe an HRM technique to detect *pncA* mutations and compare these data to sequencing and phenotypic PZA susceptibilities. This method, which involves simultaneous amplification of 7 overlapping fragments, detects *pncA* mutation within 2 hours using only a real-time PCR machine.

MATERIALS AND METHODS

Mycobacterial strains and culture conditions

Mycobacterial strains used in this study included clinical isolates and *M. tuberculosis* H37Rv (ATCC 27294). Tb isolates were cultured on Lowenstein-Jensen medium at 37°C for 2–3 weeks. Cell suspensions were prepared in Middlebrook 7H9 (M7H9) broth supplemented with Middlebrook OADC enrichment (Difco, Livonia, MI, USA) and adjusted to 0.5 McFarland for MGIT PZA susceptibility assay and DNA extraction. A total of 98 clinical isolates including 82 from Thailand (Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok), 15 from Tanzania (from Kilimanjaro Clinical Research Institute, Moshi, Kilimanjaro, Tanzania), and 1 from University of Virginia were used. Isolates were chosen on the basis of having been tested for PZA susceptibility by MGIT (2 did not grow in MGIT) towards a goal of approximately 50 susceptible and resistant isolates for assay development purposes. All work was approved by

the University of Virginia Institutional Biosafety Committee and Human Investigation Committees.

MGIT pyrazinamide susceptibility assay

PZA susceptibility tests were carried out in MGIT PZA medium (BD, Franklin Lakes, NJ, USA) according to manufacturer's protocol. Briefly, a 0.5 McFarland suspension was diluted 1:5 and 1:50 in sterile distilled water and dilutions of 1:50 were inoculated into MGIT PZA medium plus supplement without drug, dilution 1:5 were inoculated into MGIT PZA medium plus supplement with 100 μg/ml PZA drug and incubated in MGIT instrument at 37°C. Results were read automatically within 14 days after inoculation of media. *M. tuberculosis* H37Rv, susceptible to PZA, was used for quality control. All mixed population isolates were tested for PZA susceptibility twice for confirmation.

Sequencing of the *pncA* gene

DNA was isolated from 2–3 weeks of Tb culture. Briefly, 200 μl of 0.5 McFarland suspension in sterile distilled water was transferred to 2 ml screw cap tube, heat inactivated at 100°C for 30 min, and DNA extracted using DNeasy Blood & Tissue kit (Qiagen Inc, Valencia, CA, USA) according to manufacturer's protocol. The *pncA* gene was amplified by PCR using the forward primer 5′-GGTCATGTTCGCGATCGTCG-3′ and reverse primer 5′-ACAGTTCATCCCGGTTCGGC-3′ of Campbell et al[32]. Each 25-μl PCR mixture contained 12.5 μl HotStarTaq master mix (Qiagen Inc, Valencia, CA, USA), 0.15 μl of each forward and reverse 50 μM primers, 7.2 μl nuclease free water, and 5 μl of genomic DNA. PCR was performed on a MyCycler (Bio-Rad, Hercules, CA, USA) with initial denaturation at 95°C for 15 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, with a final extension at 72°C for 7 min. PCR products were analyzed on 2% agarose-gels, verified PCR products were purified using MinElute® PCR Purification Kit (Qiagen Inc, Valencia, CA, USA), measured spectrophotometrically, diluted with nuclease free water, mixed with primers and submitted to GeneWiz (GeneWiz Inc; South Plainfield, NJ, USA) for DNA sequencing.

Real-time PCR and High Resolution Melt Analysis

Seven pairs of overlapping primer were designed using Primer3 to cover upstream and the entire 561 bp *pncA* open reading frame (Table 1). Each primer pair (0.15 μl of each forward and reverse primer, 50 μM stock) was utilized in singleplex amplifications, with 25 μl PCR mixture containing 12.5 μl Type-it HRM PCR mastermix (Qiagen Inc, Valencia, CA, USA), 7.2 μl nuclease free water, and 5 μl of genomic DNA. Real time PCR was performed on Rotor Gene Q (Qiagen Inc, Valencia, CA, USA) including an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 10 sec, followed by final extension step at 72°C for 2 min. For high resolution melt fluorescence was acquired using the green channel (Excitation 470 ± 10nm, Detection 510 ± 5 nm). First, heteroduplex formation occurred for 30 sec at 50°C, followed by melt with 0.1°C increments for 2 seconds each from 84–94°C for *pncA*1, *pncA*2, and *pncA*7 primer pairs, from 81–91°C for *pncA*3, *pncA*5, and *pncA*6 primer pairs, and 79–89°C for *pncA*4 primer pairs. The reference *M. tuberculosis* H37Rv was included in each run as a wild-type positive control and nuclease free water was used for negative control.

High resolution melt analysis

Melt curve data were analyzed and normalized with the Rotor-Gene Q software. The software analyzes the difference in the shape and temperature shifts of the melt curve of the test sample from that of *M. tuberculosis* H37Rv. HRM curves were analyzed by selecting

two normalization regions, one occurring prior to the melt of the amplicon and one following complete separation of the two strands. Normalization region 1 was set at 87–87.5 °C and normalization region 2 was set at 91.5–92 °C for *pncA*1, *pncA*2, and *pncA*7 primer pairs. For *pncA*3, *pncA*5, and *pncA*6, the normalization regions were set at 84–84.5 °C and 88.5–89 °C for normalization region 1 and 2 respectively, and 82–82.5°C and 86.5–87 °C for *pncA*4. The reference *M. tuberculosis* H37Rv was set as a wild-type genotype control and percent confidence was set at 70% for all amplicons where ≥ 70 % similarity to *M. tuberculosis* H37Rv was automatically categorized as wild-type and < 70% confidence was categorized as variation.

RESULTS

Comparison of HRM analysis with Sanger sequencing

HRM analysis of the *pncA* gene was performed on DNA from 98 *M. tuberculosis* clinical isolates using 7 overlapping fragments. Representative normalized melt curves of *pncA*1, *pncA*2, *pncA*3, *pncA*4, *pncA*5, *pncA*6, and *pncA*7 are shown in Figure 1A, B, C, D, E, F, and G respectively. Each line indicates the melt curve profile for an individual sample. The normalized graph shows melt temperature shifts versus wild-type amplicon, with leftward variation indicating a lower melt temperature (e.g., C→A, C→T, G→A, G→T) and a rightward variation indicating a higher melt temperature (e.g., A→C, T→C, A→G, T→G). The difference graph uses the same data but plots the negative first derivative (−dF/dt) on the Y axis. We set the HRM software to define variation as <70% similarity with wild-type sequence.

The performance of HRM analysis was compared with Sanger sequencing as shown in Table 2. Interrogation of the entire *pncA*1-*pncA*7 fragment by HRM yielded 94% (92/98) concordance with sequencing. The HRM analysis of *pncA*1 yielded 100% concordance with sequencing of that region, while *pncA*2, *pncA*3, *pncA*4, *pncA*5, *pncA*6, and *pncA*7 yielded 88%, 94%, 95%, 95%, 96%, and 97% respectively. The majority of discrepancies were sequence mutations that fell in the primer regions and were not detected by HRM, hence the rationale for the incorporation of overlapping fragments (e.g., mutations in the forward or reverse primer regions of *pncA*2 could also be detected via the *pncA*1 or *pncA*3 amplicons, respectively). Of the 6 discrepancies, 2 were mutations detected by sequencing categorized as wild-type by HRM, specifically a C to G transversion at position 169 and a G deletion at position 290. The 4 remaining discrepancies were wild-type by sequencing but considered variation by HRM analysis. Further examination these 4 isolates showed slight variation curves on HRM, localized to *pncA*3 segment for isolate 1 and *pncA*5 segment for isolate 2, 3, and 4. Sequencing revealed mixed traces within these fragments, with both wild-type and mutant sequences present (Figure 2), suggesting that HRM was in fact accurate.

Correlation of genotypic and phenotypic pyrazinamide resistance

The genotypic sequencing and HRM analysis were compared with phenotypic MGIT PZA susceptibility results as shown in Table 3. Two isolates did not grow in the MGIT control media thus phenotypic susceptibility results were not available. Sequencing of *pncA* was concordant to phenotypic PZA susceptibility testing for 79/96 isolates (82%). Among discrepancies, 15/17 were PZA resistant while sequencing detected no mutation, and 2 were PZA susceptible with sequencing mutations, both of which were silent mutations at Ser65Ser.

HRM analysis was concordant with phenotypic PZA susceptibility for 81/96 isolates (84%). Among discrepancies, similar to sequencing, 13/15 were PZA resistant while HRM detected no variation. The 2 PZA susceptible isolates with the silent mutation Ser65Ser were falsely

categorized as variation by HRM. Overall, therefore, there were 2 fewer discrepancies with HRM than sequencing. This owed to detection of PZA resistant isolates that had mixed wild-type/variation *pncA* by HRM but were purely wild-type by sequencing.

The *pncA* mutation profiles

The 31 different *pncA* mutations found in this study are shown in Supplemental Table 1. Nine are new mutations not reported in previous studies.[33] The mutations found in *M. tuberculosis* isolates obtained from Thailand, Tanzania, and Virginia were different from each other. *pncA* mutations were observed in 35/82 (43%) Thailand isolates, the most frequent being Ile31Thr (T92C; n=8). Asp12Asn, Tyr103Stop, Val139Gly, and Thr142Met were found in 2 isolates each, and another 19 mutation profiles were found in 1 isolate of each. For Tanzanian isolates, 12/15 (80%) revealed *pncA* mutation: 5 were silent mutations at Ser65Ser, 2 with Glu111Stop, 2 with Ser65Ser/Val128Gly, and 1 each with Asp49Gly, Val169Ala, Ser179Ile. The one isolate from Virginia had a position 290 G deletion.

DISCUSSION

The current liquid-based culture systems for PZA susceptibility are complicated by their requirement for acidified media and cost not to mention turnaround time. These are particular problems for resource-limited areas, which may not have adequate susceptibility testing facilities yet may have a high burden of MDR-TB. The WHO currently recommends universal inclusion of PZA in MDR-TB regimens, however the quality of evidence for this recommendation is low, and is largely driven by the poor reproducibility of the conventional PZA method.[1, 34] In such places, a rapid genotypic-based method would be highly desirable. Unfortunately, whole length sequencing requires costly sequencing equipment. Outsourcing the sequencing to a commercial vendor is a less costly option (around 8–12 $/sample in the US) but these services are generally located in richer countries, thus would require expensive shipping from resource-limited settings. By contrast, real-time PCR platforms with HRM capabilities are becoming fairly widespread and the reagent cost of this assay is around 0.8 $/reaction or 6 $/sample. As such, this HRM method may be useful and provide substantial clinical value.

This HRM yielded 94% concordance to standard genotypic sequencing. Discrepancies were often mixed populations detected by HRM but not sequencing. The 82–84% correlation with phenotypic PZA susceptibility was expected, as this degree of correlation has been observed in larger studies.[32] The major discrepancies between HRM (or sequencing) and phenotypic susceptibility results were due to 11 PZA-resistant isolates that were wild-type by *pncA* sequencing or HRM. We repeated the MGIT method on these isolates and only 2 remained resistant and 9 were susceptible on re-assay. Such potential false resistance by MGIT method has been reported.[8–10] The two other isolates presumably have resistance mechanisms beyond *pncA* such as efflux or mutations in other genes such as rpsA.[35, 36] We did note limitations of our HRM assay, specifically the inability to detect transversions, silent mutations, or a small deletions.

To our knowledge, this is the first English language report of HRM for analyzing *pncA* mutations. One previous article has been published in the Chinese literature which utilized the Roche LightCycler 480.[37] Of note we evaluated this assay on other cyclers (Bio-Rad CFX and ABI Viia7) but found the Rotor-gene curves most easy to interpret (data not shown). Some HRM assays for Isoniazid or Rifampin include spiking of specimens with a susceptible strain such as H37Rv – we considered this but decided against it because this obliterates the ability to detect heteroresistance, a feature that was seen in some of our isolates and that we feel is relevant.

Ultimately, the utility of any *pncA*-based assay will depend on whether ~85% accuracy versus phenotypic susceptibility is acceptable. This answer will require extensive clinical study, but for the time being we think it likely is. We report the exact *pncA* mutations in the Supplemental table because growing the genotypic-phenotypic database is important for this effort of understanding the extent that genotypic information can inform phenotypic results.

CONCLUSIONS

We report a high resolution melt curve *pncA* genotypic susceptibility method to determine PZA susceptibility. The method can be used at any laboratory that has the Rotor Gene real-time PCR platform to rapidly screen PZA susceptibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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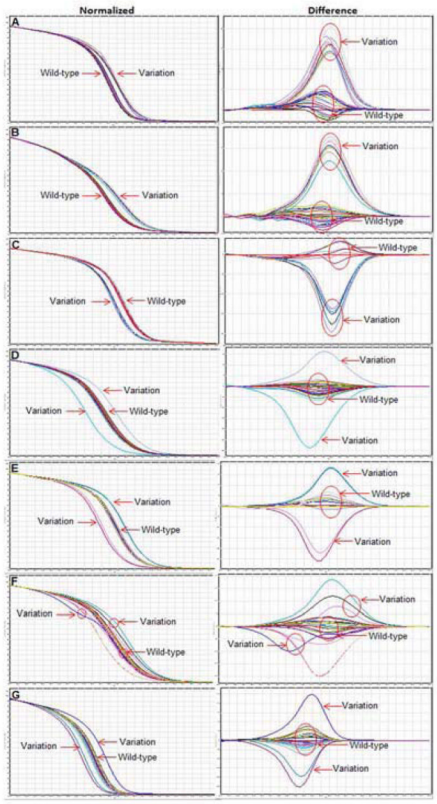

FIG. 1.

High resolution melt analysis of the entire *pncA* gene and upstream regions. Normalized (left panel) and difference graphs (right panel) of 7 amplicons are shown: *pncA*1 (A), *pncA*2 (B), *pncA*3(C), *pncA*4 (D), *pncA*5 (E), *pncA*6 (F), and *pncA*7 (G). Each line indicates the melt curve profile for an individual sample. In the difference plot, the melt curve profile of M. tuberculosis H37Rv was compared with the curve profiles of all other samples. The baseline represents M. tuberculosis H37Rv and other wild-type isolates. Isolates with "Variation" or mutant profiles, as determined by HRM software as <70% similar to wild-type, are shown.

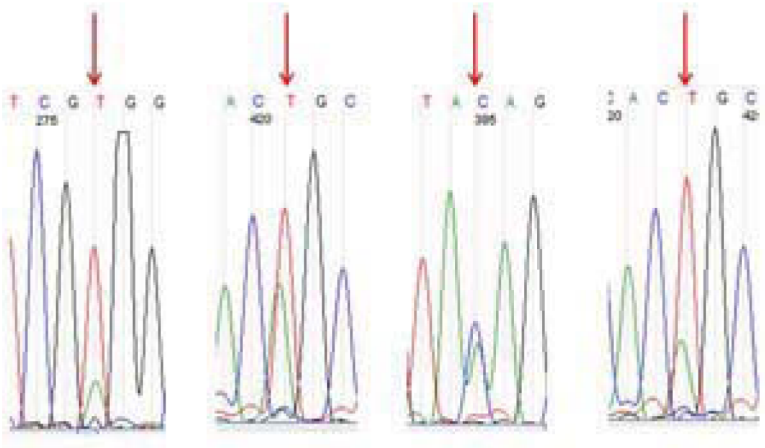

FIG. 2.
Discrepancies by sequencing and HRM. HRM showed variation for 4 samples whose sequencing revealed mixed population of sequences (arrows).

TABLE 1

Primer sequences used to amplify *pncA* gene of *M. tuberculosis* in this study.

Segment	Primers	Position	Sequences	Product size (bp)
1	*pncA*1-F	(−80)–(−61)	5′-GGCGTCATGGACCCTATATC-3′	217
	*pncA*1-R	137-119	5′-GCCACGACGTGATGGTAGT-3′	
2	*pncA*2-F	21–39	5′-CGACGTGCAGAACGACTTC-3′	179
	*pncA*2-R	199-180	5′-ACGAGGAATAGTCCGGTGTG-3′	
3	*pncA*3-F	136–155	5′-GCAACCAAGGACTTCCACAT-3′	166
	*pncA*3-R	301-281	5′-CGGTGTAGGCACCCTTGTAG-3′	
4	*pncA*4-F	236–254	5′-CGGACTTCCATCCCAGTCT-3′	103
	*pncA*4-R	338-320	5′-CCGTTCTCGTCGACTCCTT-3′	
5	*pncA*5-F	266–285	5′-CAATCGAGGCGGTGTTCTAC-3′	150
	*pncA*5-R	415-395	5′-CACAATGATCGGTGGCAATA-3′	
6	*pncA*6-F	373–392	5′-GTCGATGAGGTCGATGTGGT-3′	118
	*pncA*6-R	490-472	5′-ACACACCCGCTGTCAGGT-3′	
7	*pncA*7-F	396–415	5′-TATTGCCACCGATCATTGTG-3′	167
	*pncA*7-R	562-543	5′-ATCAGGAGCTGCAAACCAAC-3′	

TABLE 2

Performance of High resolution melt analysis comparing to Sanger sequencing.

pncA gene segment	HRM	Sequencing (n)		Accuracy (%)
		No mutation	Mutation[e]	
Entire *pncA*1–7	Wild-type	50	2[cd]	93.9
	Variation	4[a]	42	
*pncA*1	Wild-type	82	0	100
	Variation	0	16	
*pncA*2	Wild-type	76	12[bc]	87.8
	Variation	0	10	
*pncA*3	Wild-type	83	5[bc]	93.9
	Variation	1[a]	9	
*pncA*4	Wild-type	91	3[bd]	94.9
	Variation	2[a]	2	
*pncA*5	Wild-type	87	2[bd]	94.9
	Variation	3[a]	6	
*pncA*6	Wild-type	87	3[b]	95.9
	Variation	1[a]	7	
*pncA*7	Wild-type	86	3[b]	96.9
	Variation	0	9	

[a] mixed population with wild-type predominant in sequencing trace file,

[b] mutation at HRM primer region,

[c] transversion C to G,

[d] G deletion,

[e] the total number of isolates with mutations identified in the (entire) pncA amplicon (n=44) is less than the number of isolates with mutations identified in the 7 pncA amplicons because these 7 amplicons overlap and will double-count mutations in overlapping regions. For example 8 isolates contained mutation at position 92 which was detected by both pncA1 primer pair ((−80) to137) and pncA2 primer pair (21 to 199).

TABLE 3

Assay performance of Sequencing and HRM compared to MGIT PZA susceptibility test.

Sequencing	MGIT PZA susceptibility test (n)		Accuracy (%)	HRM	MGIT PZA susceptibility test (n)		Accuracy (%)
	Susceptible	Resistant			Susceptible	Resistant	
No mutation	39	15[a]	82.3	Wild-type	39	13[b]	84.4
Mutation	2[c]	40		Variation	2[c]	42	

[a] 11 isolates had no mutation and remaining isolates had mixed populations but were wild-type predominant

[b] 11 isolates were no-mutation, 1 isolate was a C to G transversion, 1 isolate was a 290 G deletion,

[c] 2 isolates had silent mutation Ser65Ser