Next-Generation Ion Torrent Sequencing of Pyrazinamide Resistance in MDR/XDR South African *Mycobacterium tuberculosis* Strains

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INTRODUCTION

Pyrazinamide (PZA) is an important first-line antibiotic and may play an essential role in treating multidrug resistant (MDR) and extensively drug resistant (XDR) *Mycobacterium tuberculosis* (MTB). Phenotypic drug susceptibility testing (DST) is considered the gold-standard for resistance detection. However, it is time consuming (weeks to months) and technically challenging since MTB growth is impeded by acidic conditions required for PZA drug activity. Additionally, molecular detection of PZA resistance is complicated because the 561-bp *pncA* gene contains a variety of substitution, insertion, and deletion mutations that confer resistance. Previously we described an Ion Torrent sequencing procedure for analysis of five MTB resistance genes from cultures using full-length gene amplification primers[1].

SPECIFIC AIMS

In this study we describe the application of a rapid and simplified library preparation procedure that utilizes a tiled, overlapping, full-gene amplification approach using next-generation Ion Torrent sequencing. This tiled approach circumvents DNA shearing (chemical or physical) and size-selection steps involved in next-generation library preparation. This approach was used for genetic characterization of the full-length *pncA* gene from a selection of South African isolates and primary sputum specimens.

METHODS

Geographically diverse MDR and XDR isolates and primary sputum specimens from South Africa were collected, inactivated, and shipped at ambient temperature in PrimeStore MTM®. DNA was extracted and full-length *pncA* genes were amplified by a tiled approach (Figure 1A), sequenced using the Ion Torrent (Figure 1B), and compared to results using the BACTEC™ MGIT™ 960.

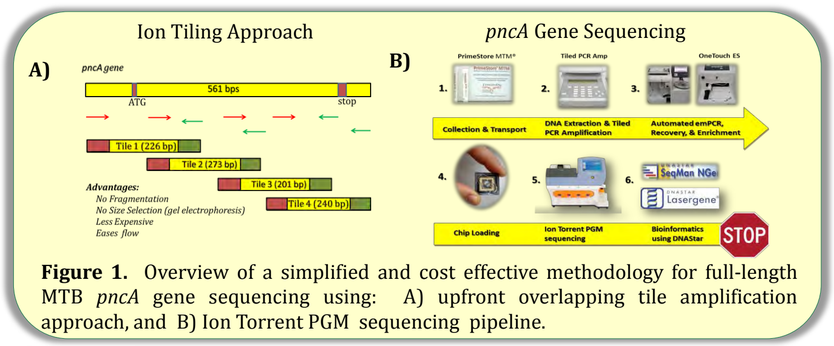

Figure 1. Overview of a simplified and cost effective methodology for full-length MTB *pncA* gene sequencing using: A) upfront overlapping tile amplification approach, and B) Ion Torrent PGM sequencing pipeline.

RESULTS

A limit of detection using a 10-fold serially diluted *M. tuberculosis* strain was performed for each *pncA* gene tile and compared to real-time PCR (Figure 2). Four *pncA* gene tiles (Tiles 1-4) ranging from 201 to 274 bp comprising the full-length pncA coding region were visualized across 4-logs, equating to ~8 x 10^3 CFU/mL MTB (Figure 2). An MTB dilution of 8 x 10^3 CFU/mL corresponded to a real-time PCR cycle threshold (C_T) value of 30.9 (Table 1).

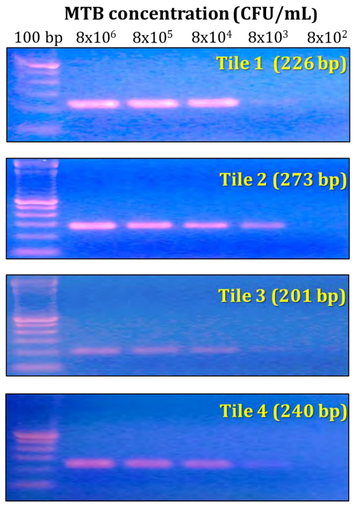

Figure 2. A tiled PCR amplification from a 10-fold serially diluted *M. tuberculosis* strain. Four *pncA* gene tiles (Tiles 1-4) ranging from 201 to 274 bp comprising the full-length *pncA* coding region were visible across a 4-log reduction using UV and 2% ethidium bromide-stained agarose gels. Bands for all 4 *pncA* amplicon tiles were noted at ~8 x 10^3 CFU/mL with no visible amplicons detected at 8 x 10^2 CFU/mL. The 8 x 10^3 CFU/mL corresponds to a real-time PCR cycle threshold (C_T) value of 30.9. Positive and negative control reactions were performed for each *pncA* gene tile. 100 bp DNA ladder is shown.

This simplified, tiled methodology, *pncA* gene amplification was performed using MTB cultures and primary sputum specimens from South Africa (Table 1). Using real-time PCR, a primary sputum specimen quantitative cycle threshold value of 30 or less ($C_T \leq 30$) was a positive indicator for successful gene amplification (Table 1). Several known and novel PZA-resistant mutations were identified from MDR/XDR culture isolates and primary specimens that were confirmed by DST testing (Table 2).

TABLE 1. Tiled PCR amplification of *the pncA* gene from positive South African clinical isolates and primary sputum specimens compared to quantitative real-time PCR cycle threshold values.

Sample ID	Real-time PCR cycle threshold value (C_T)[†]	Positive PCR amplification of *pncA* gene (tiles 1-4)	Full gene Ion Torrent sequencing
37 clinical isolates	13.9 to 21.1	Yes	Yes
5 NALC[¥] processed sputum specimens	21.1 to 25.9	Yes	Yes
2 primary sputum specimens	27.8 and 28.5	Yes	Yes
7[‡] primary sputum specimens	36.0 to 40*	No	No
Positive Control (8x10^5 CFU/mL)	23.1	Yes	Yes
Positive Control (8x10^4 CFU/mL)	25.1	Yes	Yes
Positive Control (8x10^3 CFU/mL)	27.5	Yes	Yes
Positive Control (8x10^2 CFU/mL)	30.9	Yes	Yes
Positive Control (8x10^1 CFU/mL)	36.8	No	No

[†] *Triplicate reactions were performed*
* *A cycle threshold (C_T) value of 40 indicates No Detection.*
[‡] *3 of 7 primary sputum specimens were not detected (C_T=40) by real-time PCR. The remaining 4 exhibited C_T values of 36.0, 36.4, 36.6, and 38.1.*
[¥] *Specimens processed by N*-acetyl-l-cysteine-sodium hydroxide (NALC).

RESULTS

TABLE 2. Summary of amino acid mutations in the *pncA* gene of *M. tuberculosis* clinical isolates and primary sputum specimens from South Africa deduced by tiled amplification and Ion Torrent Sequencing.

Number of samples and type	Amino acid substitution(s)[†] yielded by the *pncA* gene (561 bp)	Pyrazinamide result: Ion Torrent[‡]	Pyrazinamide result: Bactec MGIT 960
37 clinical isolates:			
3	**C14R**	Resistant	Resistant
1	**A102V**	Resistant	Resistant
1	**Q122STOP**	Resistant	Resistant
21	Wild Type[†]	Sensitive	Sensitive
1	**V139G**	Resistant	Resistant
1	**R154G**	Resistant	Resistant
2	**L172P**	Resistant	Resistant
1	Silent (C195T)[¥]	Sensitive	Sensitive
1	**D12G***	Resistant	Resistant
1	**H51D, H71Y***	Resistant	Resistant
1	**S59P, H71Y***	Resistant	Resistant
1	**A79V***	Resistant	Resistant
1	**K96STOP***	Resistant	Resistant
1	**T135P***	Resistant	Resistant
5 NALC original specimens:			
5	Wild Type[†]	Sensitive	Sensitive
2 primary sputum samples:			
2	Wild Type[†]	Sensitive	Sensitive

[†] *compared to the H37Rv reference strain.*
[‡] *Pyrazinamide resistance is known to occur in several mutations described by Mphahlele et al. (2) (indicated in bold).*
[¥] *One strain contained a silent (synonymous) nucleotide mutation corresponding to position 195 (C →T).*
**There is a heterozygous nucleotide mutation in a proportion of Ion Torrent reads; the mutation confers a mixed amino acid substitution.*

CONCLUSIONS

- This rapid Ion Torrent tiled amplification and sequencing approach does not entail mechanical or chemical DNA shearing or size selection (Figure 1). Therefore, expensive equipment, i.e., gel electrophoresis, Pippin Prep Workstation, and the DiaGenode Bioruptor are not required.
- Using this approach, library preparation for next-generation Ion Torrent sequencing is less tedious and timely, i.e., <1 day of preparation, and reduces the overall cost of sequencing. DNA extraction to sequence analysis is less than 2 days (Figure 1).
- A real-time PCR assay for universal detection of MTB complex from samples with a quantitative cycle threshold value of 30 or less (CT ≤ 30) was a positive indicator for successful gene tile amplification using our rapid sequencing approach (Figure 2).
- The reduced workflow and increased sensitivity of this approach allows for genetic analysis of the MTB *pncA* gene directly from primary sputum samples (Table 2).
- Full-length *pncA* gene analysis from South African isolates and primary specimens contain a complex variety of mutations that can be used to determine PZA resistance and may also be used to track and monitor the acquisition of new resistance mutations in circulating strains (Table 2).

REFERENCES

1. Daum LT, Rodriguez JD, Worthy SA, Ismail NA, Omar SV, Dreyer AW, Fourie PB, Hoosen AA, Chambers JP, Fischer GW. 2012. Next-generation ion torrent sequencing of drug resistance mutations in *Mycobacterium tuberculosis* strains. *Journal of Clinical Microbiology*. Dec; 50(12): 3831-3837.

2. Mphahlele M, Syre H, Valvatne H, Stavrum R, Mannsaker T, Muthivhi T, Weyer K, Fourie PB, and Grewal HMS. 2008. Pyrazinamide Resistance among South African Multidrug-Resistant *Mycobacterium tuberculosis* Isolates. *Journal of Clinical Microbiology*, 46(10): 3459-3464.

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