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Rapid Sequencing of the *Mycobacterium tuberculosis pncA* Gene for Detection of Pyrazinamide Susceptibility

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We developed a pyrazinamidase gene DNA-sequencing method to rapidly identify pyrazinamide resistance-causing mutations in GenoLyse-treated, smear-positive sputum specimens. The sensitivity and specificity were 90.9 and 100%, respectively, compared to those of MGIT drug susceptibility testing, after the exclusion of synonymous mutations and nonsynonymous mutations previously associated with susceptibility to pyrazinamide.

Tuberculosis (TB) clinical trials are planned for the evaluation of new treatment regimens that include pyrazinamide (PZA); however, culture-based methods for the detection of PZA resistance are complicated because: pyrazinamidase (PZase) is active at low pH only, results are influenced by inoculum size, and the assays are difficult and time-consuming (1, 2). Alternative methods to rapidly screen for PZA susceptibility need to be evaluated. Point mutations in the *pncA* gene, which encodes PZase, are the primary mechanism of resistance to PZA (3, 4); however, mutations and/or polymorphisms occur across the entire length of the gene, and thus, sequencing of the entire *pncA* gene is necessary to capture all of the possible mutations (5). Here we evaluated *pncA* DNA sequencing to detect PZA resistance-conferring mutations directly in clinical specimens.

To determine the diagnostic performance characteristics of *pncA* gene sequencing, a set of 85 of 167 stored, GenoLyse-treated specimens from the NC-002 clinical trial (TB Alliance) were selected because these specimens also had MGIT PZA drug susceptibility data (Becton, Dickinson and Company, NJ, USA). All 85 GenoLyse-treated specimens had been stored on their pellets, contrary to the manufacturer's instructions (Hain Lifescience, Germany). Ethical approval was obtained from the Health Research Ethics Committee of Stellenbosch University, Pharma Ethics, University of Witwatersrand and University of Cape Town.

The *pncA* gene was amplified with primers JpncAs Forward (5′ GGCGTCATGGACCCTATA 3′) and JpncAs Reverse (5′ GTGA ACAACCCGACCCAG 3′). These primers were designed to amplify a product of 738 bp that includes the full length of the *pncA* gene (561 bp), as well as 80 bp of the upstream sequence and 97 bp of the downstream sequence. Each reaction mixture (25 μl) contained 1 to 2.5 μl of template DNA (GenoLyse-treated sputum specimen), 0.75 μM each PCR primer (IDT), 1× buffer, 0.8 mM deoxynucleoside triphosphates, 0.125 μl (0.625 U) of HotStarTaq polymerase (Qiagen, Germany), and 2 μM Syto9 green-fluorescent nucleic acid stain (Invitrogen, CA, USA). The amplification protocol consisted of an initial activation step of 95°C for 15 min, followed by 50 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min and a final elongation step of 10 min at 72°C. Water was included as a negative control, while H37Rv DNA was included as a positive control. Amplification was confirmed by the detection of a distinct fluorescent melting curve signature consisting of two maxima (±92°C and ±93.5°C, respectively) by high-resolution melting analysis. Post-PCR cleanup was done with the Agencourt AMPure XP PCR1 purification system (Agencourt Bioscience Corporation, Beckman Coulter, Beverly, MA). PCR products were sequenced in both directions with the JpncAs Forward and JpncAs Reverse primers. The DNA sequencing reaction was done on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following program: 5 min at 94°C, followed by 25 cycles of 94°C for 10 s, 55°C for 10 s, and 60°C for 4 min, using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Austin, TX, USA). The products were then electrophoresed on an ABI3730xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed on the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html) with H37Rv as the reference strain. The sensitivity and specificity of the DNA sequencing method were determined with Statistica 7.1.

Table 1 shows that 78 (91.8%) specimens were amplifiable and gave interpretable DNA sequences. The absence of amplification in the remaining seven (8.2%) specimens was ascribed to the storage of the GenoLyse extracts on their pellets. *pncA* sequences were available for 11 of 13 samples with a resistance phenotype (1 had a wild-type sequence, and 10 had nonsynonymous mutations) (see Table S1 in the supplemental material). *pncA* sequences were available for 67 of the 72 PZA-susceptible samples (2 had synonymous mutations, 4 had nonsynonymous mutations, and 61 had wild-type sequences [see Table S1 in the supplemental material]). Review of the four nonsynonymous mutations showed that they

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TABLE 1 Comparison of *pncA* sequencing results and Bactec MGIT 960 PZA drug susceptibility test results

Bactec MGIT PZA drug susceptibility test or *pncA* sequencing result	No. (%) of samples
PZA resistant ($n = 13$)	
pncA nonsynonymous mutation	10 (76.9)
pncA wild type	1 (7.7)
PCR negative	2 (15.4)
PZA susceptible ($n = 72$)	
pncA wild type	61 (84.7)
pncA synonymous mutation	2 (2.8)
pncA nonsynonymous mutation	4[a] (5.6)
PCR negative	5 (6.9)

[a] All previously associated with low-level PZA phenotypic resistance (6–10).

had been previously associated with PZA susceptibility or low-level resistance (V130A, L35R, and T114M) (6–10). Thus, a total of 77 (98.7%) of the 78 specimens would have been correctly scored on the basis of an algorithm that incorporates previously described genotype-phenotype relationships. This translates to a sensitivity and specificity of 90.9% (95% confidence interval [CI] = 0.739 to 1.079) and 100% (95% CI = 1.0), respectively, assuming that the MGIT susceptibility results were correct. The negative and positive predictive values were 0.985 and 1.000, respectively.

Our turnaround time for PCR and DNA sequencing was 43 h, compared to conventional culture's 26 days (range, 12 to 195 days). This shortened time to diagnosis will ensure that patients with a high risk of PZA resistance (multidrug-resistant TB [MDR-TB]) are not exposed to inappropriate therapy for an extended period and can be excluded from trials where PZA is central to therapy.

The diagnostic value of the DNA-sequencing method relies on our knowledge of the relationship between the genotype and the phenotype. Review of the current literature shows that 87 to 95% of the isolates determined phenotypically to be PZA resistant harbor nonsynonymous mutations in the *pncA* gene (5). PZA resistance in the remaining 5 to 13% of the isolates may occur through mutations in other genes, including the *rpsA* gene (11, 12) or could be false positives of the phenotypic assays (2, 13). Inclusion of the *rpsA* gene may improve the assay. Mutations in *pncA* are not restricted to PZA-resistant isolates, and synonymous and nonsynonymous mutations have been documented in PZA-susceptible isolates. Pending a full, definitive, quantitative understanding of the relationship between *pncA* mutation and PZA susceptibility, we propose that wild-type sequences should be considered susceptible with a low risk of PZA resistance through alternative mechanisms. Mutations in *pncA* should be considered resistant unless synonymous or previously proven to not confer resistance.

Two additional limitations were noted. First, correct storage of GenoLyse DNA extracts is essential for consistent PCR amplification. This can be overcome by aspiration of the extracted DNA into a clean tube, followed by storage at −80°C, as recommended by the manufacturer. Second, the DNA sequencing method was tested on DNA extracts from smear-positive sputum samples only, and it is therefore not plausible at this time to extrapolate these findings to smear-negative sputum samples.

In summary, we have demonstrated that targeted DNA sequencing is a simple, rapid, and accurate method for detecting PZA resistance that is suitable for clinical trials, as well as for routine testing of MDR-TB cases.

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