Pyrazinamide Resistance in *Mycobacterium tuberculosis*

by

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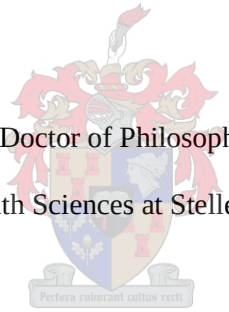

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for degree.

This dissertation includes 3 original papers published in peer-reviewed journals or books and 2 unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Signature …… Date ……

Abstract

Pyrazinamide (PZA) is one of the drugs included in the standardized TB treatment regimen for both drug susceptible and resistant TB. PZA has a unique ability to target persister bacilli and since its inclusion has reduced TB treatment duration to 6 months. However, drug susceptible testing (DST) for PZA is not routinely performed due to the challenges associated with the DST (acidic media; false resistance and inoculum size). Significant evidence indicates that mutations in *pncA* (gene encodes for pyrazinamidase (PZase)) are the primary mechanism of resistance to PZA. Sequencing studies reveal that mutations occur across the entire length of the gene, thus sequencing of the entire gene is required to capture all possible resistance conferring mutations. A rapid molecular diagnostic is required to routinely test for PZA resistance, especially given the continued recommendation of its inclusion in the shortened MTB treatment regimen.

PZA is currently being considered for inclusion of new treatment regimens which also include the newer anti-TB drugs. However, we need to better understand the prevalence of PZA resistance globally and across the spectrum of drug resistant groups to determine the utility of this drug in current and future treatment regimens. This PhD thesis aims to further the current knowledge of PZA resistance.

The prevalence of PZA resistance regionally and globally is largely unknown. Our systematic review collates the PZA resistance data in the literature from all TB cases (16.2%); in high-risk MDR-TB (41.3%) and in MDR-TB (60.5%). These findings caution against relying on PZA in current and future TB drug regimens, especially in MDR-TB patients. This review also identified more than 600 unique SNPs across the entire length of the *pncA* gene as the causal mechanism of resistance. This highlights the complexity of the challenge to develop future genetic based PZA resistance tests.

From this review it was evident that the prevalence of PZA resistance across a spectrum of drug resistant isolates is unknown. To address this knowledge gap 775 clinical isolates from South Africa were classified into groups according to resistance profile (ranging from pan-susceptible to XDR-TB) and their association with PZA resistance was determined. The prevalence of PZA resistance in each group was: 0% in the pan-susceptible; 2% in the INH-mono; 7.5% in the RIF-mono; 39.3% in the MDR-TB and 96.8% in the XDR-TB group. A statistically significant increase in PZA resistance was

observed as the number of resistance markers increases. We concluded that PZA DST should be performed when considering its inclusion in treatment of patients with rifampicin-resistant TB or MDR-TB. This study also showed an excellent correlation between *pncA* genotype and PZA phenotype. The resulting sensitivity and specificity was 95% and 99%, respectively, thereby confirming the utility of *pncA* sequencing as a rapid PZA susceptibility test.

In an attempt to understanding discordance between genotype and phenotype we characterised *pncA* mutations which did not confer PZA resistance at a susceptibility breakpoint of 100 µg/ml pyrazinamide in MGIT. A total of 10 non-resistance conferring mutations were identified in collaboration with the U.S. Centers for Disease Control and Prevention. These results will be essential for the interpretation of *pncA* sequencing results to guide treatment.

In a recent paper the authors suggested that *pncA* mutation have a fitness cost which would impact on the transmission of drug resistant *M. tuberculosis* strains. To test this hypothesis we compared the growth rates of clinical isolates and *in vitro* mutants (wild type and SNPs) and clinical isolates which have a large *pncA* deletion. No difference was observed in the time to positivity in MGIT media when comparing wild type and strains harbouring *pncA* mutations, thereby suggesting that *pncA* mutation do not have a significant fitness cost. However, strains harbouring large *pncA* mutations showed a significant growth deficit (p-value: <0.001) *in vitro*. However, this fitness cost did not prevent these strains from transmitting in the community.

In an attempt to simplify the diagnosis of PZA susceptibility, we investigated the diagnostic utility of a LATE-PCR based technique in combination with fluorescence probes to identify mutations along the entire length of the *pncA* gene. Using this method the sensitivity and specificity was 98.7% and 98.4%, respective, suggesting that this method could be using in routine laboratories due to the single tube format.

Together this body of research has addressed critical knowledge gaps and has introduced a methodology that could simplify the diagnosis of PZA resistance.

Opsomming

Pyrazinamide (PZA) is ‘n middel wat gebruik word in die standaard behandeling van beide sensitiewe en weerstandige tuberkulose (TB). PZA beskik oor die unieke vermoë om langlewende bakterieë te teiken en het sedert die insluiting daarvan in die behandelingsregime die duur van TB behandeling verminder tot 6 maande. Middel weerstandige toetsing (MWT) vir PZA word selde uitgevoer as gevolg van uitdagings wat geassosieer word met MWT (suur media, vals positiewe weerstandigheid en inokulum grootte). Betekenisvolle resultate het gewys dat mutasies in *pncA* (die geen wat kodeer vir pyrazinamidase (PZase)) die primêre meganisme is wat lei tot PZA weerstandigheid. Volgordebepaling het gewys dat mutasies oor die hele lengte van die geen voorkom en gevolglik moet die hele geen se volgorde bepaal word om al die moontlike mutasies wat kan bydra tot weerstandigheid te identifiseer. ‘n Vinnige molekulêre diagnostiese toets word benodig om op ‘n gereelde basis te kan toets vir PZA weerstandigheid, veral gegewe die volgehoue aanbeveling dat PZA ingesluit moet word in die verkorte TB behandeling.

PZA word tans oorweeg vir die insluiting in nuwe behandelingsregime wat ook nuwer anti-TB middels sal insluit. Ons benodig egter verbeterde kennis omtrent die globale voorkoms van PZA weerstandigheid oor die spektrum van weerstandige groepe om vas te stel wat die verbruik van die middel in die huidige en toekomstige behandeling is. Die doel van hierdie PhD tesis is om die huidige kennis van PZA weerstandigheid uit te brei.

Die plaaslike en globale voorkoms van PZA weerstandigheid is grotendeels onbekend. Ons sistematiese oorsig artikel vat die beskikbare PZA weerstandige data in die literatuur oor alle TB gevalle (16.2%), in hoë risiko multiweerstandige TB (MDR-TB) (41.3%) en in MDR-TB (60.5%) gevalle saam. Hierdie bevindinge waarsku teen die betroubaarheid van PZA in huidige en toekomstige TB behandeling, veral in MDR-TB pasiënte. Die studie het meer as 600 unieke enkelnukleotied polimorfismes oor die lengte van die *pncA* gene geïdentifiseer as die oorsaak van weerstandigheid. Dit beklemtoon die kompleksiteit van die uitdaging om toekomstige geneties gebaseerde PZA weerstandige toetse te ontwikkel.

Hierdie oorsig artikel het ook daarop gedui dat die voorkoms van PZA weerstandigheid oor ‘n spektrum van middel weerstandige isolate onbekend is. Om hierdie gebrek in kennis aan te spreek is 775 kliniese isolate van Suid-Afrika geklassifiseer in groepe volgens hul weerstandige profiele (wat strek van middel vatbare to XDR-TB) en is hul assosiasie met PZA weerstandigheid bepaal. Die voorkoms van PZA weerstandigheid in elke groep was soos volg: 0% in die middel vatbare; 2% in die INH-enkelweerstandig; 7.5% in die RIF-enkelweerstandig; 39.3% in die MDR-TB en 96.8% in die XDR-TB groep. Soos die aantal weerstandige merkers toeneem is ‘n beduidende verhoging in PZA weerstandigheid waargeneem. Ons het beveel aan dat PZA MWT uitgevoer moet word wanneer dit oorweeg word om pasiënte met rifampisien-weerstandige TB of MDR-TB ook met die middel PZA te behandel. Hierdie studie dui ‘n uitstekende korrelasie tussen *pncA* genotipes en PZA fenotipes aan. Die sensitiwiteit en spesifisiteit was onderskeidelik 95% en 99%, wat die bruikbaarheid van *pncA* volgordebepaling bevestig as ‘n vinnige PZA sensitiewe toets.

In ‘n poging om die teenstrydigheid tussen genotipe en fenotipe te verstaan, het ons *pncA* mutasies wat nie PZA weerstandigheid veroorsaak by ’n konsentrasie van 100 µg/ml nie, in meer diepte ondersoek. ‘n Totaal van 10 mutasies wat nie bydra tot weerstandigheid nie is geidentifiseer in samewerking met die VSA se Sentrum vir Gesondheidsbeheer en Voorkoming. Laasgenoemde data is krities vir die interpretasie van *pncA* volgordebepaling resultate om behandeling voor te kan skryf.

In ‘n onlangse artikel het die outeurs voorgestel dat die fiksheid koste van *pncA* mutasies ‘n impak het op die oordrag van middel weerstandige *M. tuberculosis* stamme. Om hierdie hipotese te toets het ons die groei tempo van kliniese isolate en in vitro mutasies (wilde tipe en enkelnukleotied polimorfismes) en kliniese isolate wat ‘n groot *pncA* delesie bevat vergelyk. Geen verskil is opgemerk in die tyd tot positiwiteit in MGIT media wanneer wilde tipe met die stam wat *pncA* mutasies bevat vergelyk is nie, wat daarop dui dat die *pncA* mutasies nie ‘n merkwaardige effek op fiksheid het nie. Stamme wat egter groot *pncA* mutasies bevat, het ‘n merkwaardige groei vertraging (p-waarde: <0.001) *in vitro* getoon. Hierdie effek het egter het nie die vermoë van hierdie stamme verander om oorgedra te word aan die gemeenskap nie.

In ‘n poging om die diagnose van PZA weerstandigheid te vereenvoudig het ons die diagnostiese gebruik gemaak van ‘n “LATE-PCR” gebasseerde tegniek kombineer met fluoresserende merkers om

mutasies oor die hele *pncA* geen te identifiseer. Hierdie metode se sensitiwiteit en spesifisiteit was onderskeidelik 98.7% en 98.4%, wat daarop dui dat hierdie metode gebruik kan word in roetine laboratoriums, veral omdat daar van ‘n enkel-buis formaat gebruik gemaak word.

Tesame het hierdie navorsing die kritieke gapings in kennis aangevul en het dit ‘n metode voorgestel wat die diagnose van PZA weerstandigheid kan vereenvoudig.

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List of Abbreviations

A	Adenine or adenosine, one-letter code for alanine
Bp	Base pairs
C	Cytosine or cytidine, one-letter code for cysteine
CI	Confidence interval
CO	Cal orange
CR	Cal red
DNA	Deoxyribo-nucleic acid
dNTP	Deoxynucleoside triphosphate
DOTS	Directly observed therapy short-course
DR	Drug resistance
DS	Drug susceptible
DST	Drug susceptible testing
EDTA	Ethylenediaminetetraacetic acid
EMB	Ethambutol
et al.	*et al*ii (and others)
g	Grams
G	Guanine or guanosine, one-letter code for glycine
HCl	Hydrochloride
Indels	Small insertions and deletions
INH	Isoniazid
Kb	Kilobase
LATE	Linear-after-the-exponential
M.	*Mycobacterium*
M	Molar / relative molecular weight
MDR	Multi drug resistant
mg	Milligrams
$MgCl_2$	Magnesium chloride

MGIT	Mycobacterial growth indicator tube
mM	Milimolar
mm	Millimetre
ml	Millilitre
NGS	Next generation sequencing
NHLS	National health laboratory services
nSNP	Non-synonymous single nucleotide polymorphisms
Nt	Nucleotide
PaMZ	Pretomanid + Moxifloxacin + Pyrazinamide
PCR	Polymerase reaction chain
pH	Potential of hydrogen
POA	Pyrazinoic acid
PZA	Pyrazinamide
PZase	Pyrazinamidase
QSR	Quasar
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
RNase A	Ribonuclease A
SA	South Africa
SNP	Single nucleotide polymorphism
sSNP	Synonymous single nucleotide polymorphism
T	Thymine or thymidine
TAE	Tris Acetate EDTA (buffer)
TB	Tuberculosis
TE	Tris EDTA
Tm	Melting temperatures
TraSH	Transposon site hybridization
U	Units

USA	United States of America
WGS	Whole genome sequencing
WHO	World health organization
XDR	Extensively drug resistant
°C	Degrees Celsius
µg/ml	Micrograms per millilitre
µl	Microliters
X g	Relative centrifugal force
µM	Micro molar

List of Symbols

µ	Micro
%	Percentage
™	Trademark
°C	Degrees Celsius
Δ	Delta
~	Approximately

List of Tables and Figures

Chapter 1

None

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Table of Contents

Chapter 1

General Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB) disease, an airborne infectious disease which is preventable and curable. According to the latest World Health Organization (WHO) TB report there were an estimated 9.6 million new cases of TB and 1.5 million deaths from TB globally each year (1). The current burden of TB on the health authorities can in part be attributed to drug resistance. Multi-drug resistant (MDR)-TB is defined as *M. tuberculosis* which has become resistant to at least two of front line anti-TB drugs: rifampicin (RIF) and isoniazid (INH) (1). The WHO estimates that between 360 000 and 600 000 MDR-TB cases occur among reported TB cases globally (1). Extensively drug resistant (XDR)-TB, the more severe and frequently fatal form of drug resistance, has been identified in various parts of the world, including in South Africa (2). XDR-TB is defined by the WHO as *M. tuberculosis* strains that are MDR and in addition, also resistant to a fluoroquinolone and one of the injectable drugs (kanamycin, amikacin and capreomycin). Resistance can be acquired, due to inadequate treatment (termed acquired resistance). Alternatively, resistance can be primary which means that already resistant bacilli were transmitted to the individual. Recent transmission plays a major role as the driving force of the drug resistant TB epidemic (3). It is important to control and reduce the rates of MDR-TB as it places a huge financial and public health burden on national TB control programmes world-wide (4). However, current efforts to control MDR-TB are inadequate as only 25% of those diagnosed were placed on treatment (1).

Central dogma suggests that resistance develops through a process of spontaneous mutation (synonymous single nucleotide polymorphisms (sSNPs)) in target genes, followed by antibiotic selection during periods of poor adherence or monotherapy. Resistance of *M. tuberculosis* to anti-TB drugs is thought to occur solely through mutations in target or related (e.g., pro-drug activating) genes (5). This concept is based on the close correlation of drug-resistance phenotypes with specific gene polymorphisms. Drug resistance develops spontaneously and is selected by exposure to sub lethal levels of antibiotics (6). It has been shown that resistance is due to chromosomal mutations in specific genes of *M. tuberculosis* (7). Evidence of drug resistance mechanisms has been provided by cloning and characterization of these specific genes in drug resistant isolates as compared to drug susceptible isolates (8). Thus the identification of these resistance conferring mutations has been proposed as a means to genetically identify drug resistance, in particular MDR-TB through the identification of

mutations in the *rpoB* gene (RIF), *inhA* promoter and *katG* gene (INH). Detection of mutations in these targets forms the basis for rapid diagnostics to reduce the time to diagnosis and thereby limit transmission of drug-resistance.

The anti-TB drug pyrazinamide (PZA) is an integral component of the standardized TB treatment regimen for drug susceptible and drug resistant TB recommended by the WHO (1, 9). This is because of its unique ability to eradicate persister bacilli which are not easily eradicated with the other anti-TB drugs (10, 11). The importance of PZA has recently come to the forefront because this drug has been shown to deplete membrane energy and to inhibit trans-translation (12, 13). Both of these processes are thought to be essential for the survival of drug-tolerant persistent bacilli under various stress conditions (14). The addition of PZA to the treatment regimen for TB has allowed the duration of therapy to be shortened to 6 months and this drug has been credited with the success of the current directly observed therapy short-course (DOTS) strategy (10, 11).

Diagnostic services are desperate for a molecular assay which can give rapid and accurate susceptibly and resistance information. However, the currently implemented GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA) only detects TB and RIF resistance. Currently, there is no molecular assay for PZA resistance, and routine culture-based PZA susceptibility testing is not done in many parts of the world (including South Africa) due to the technical complexity of the assay.

Pyrazinamide Susceptibility Testing Methods

Culture-Based Pyrazinamide drug susceptibility testing

Two commercial PZA drug susceptibility tests have been exhaustively evaluated: the BACTEC 460TB System Mycobacterial Culture Media and the BACTEC MGIT 960 System (Becton Dickinson, Sparks, MD). A recent comparison of these methods showed that the BACTEC 460 system was more reliable for testing for PZA resistance as the authors observed that the BACTEC 960 system reported a number of false positives (15). A factor which influenced the scoring of false positives includes acidity of the media which may be titrated upwards by inoculum size (16-18). For this reason, the WHO has not approved a standardized culture-based method for PZA DST due to the complexity and inconsistency of the currently available culture-based tests (1, 19-21).

Pyrazinamidase activity assay

This assay is based on the understanding that *pncA* encodes for the PZase enzyme. This enzyme is required to convert PZA into the active form of pyrazinoic acid (POA). It is hypothesized that mutations in the *pncA* gene substantially decrease PZase activity, thereby limiting the conversion of PZA to the active form of POA in *M. tuberculosis* (22) An PZase activity assay known as the Wayne's method was developed by Wayne in 1974 which assesses the function of the PZase enzyme based on a colorimetric change (23). A modified method was also developed, whereby the critical concentration was changed from 100 µg/ml to 400 µg/ml and a slightly different media was used. A study performed by Miller et al (1995) found a very good correlation between the radio-metric BACTEC system and PZase (classic Wayne's method) (24). They described a correlation of 98.2% and 100% in susceptible and resistant isolates respectively (24). A more recent study (2009) investigated the concordance of BACTEC 960 with both the classical Wayne's method and the modified Wayne's method (25). This study reported 88.9% concordance between BACTEC 960 and the classical Wayne's method while 93.4% concordance between BACTEC 960 and the modified Wayne's method. Both the studies show that the PZase activity assay can be used to detect PZA resistance but that resistance should be confirmed via culture-based DST. This method could be used as a screening test as suggested by the Sharma study (25).

***pncA* genotyping**

Numerous studies have demonstrated that mutations in *pncA* are the primary mechanism of resistance to PZA (26-28). However, the correlation between genotype and phenotype is not so clear-cut. Extensive deoxyribonucleic acid (DNA) sequencing studies of *pncA* have revealed that numerous mutations and/or polymorphisms, which occur across the entire length of the gene, making it a complex target for the development of a genetic-based DST to identify causal mutations (29-32). This implies that sequencing the entire *pncA* gene is essential in order to capture all the possible mutations and to differentiate between non-synonymous and synonymous single nucleotide polymorphisms (SNPs). However, the identification of causal mutations is further complicated by the fact that not all non-synonymous mutations cause phenotypic resistance (33). Furthermore, sequencing

phenotypically PZA resistant samples has shown in a small percentage of these isolates that mutations in the *pncA* gene were absent (34, 35). This suggests that resistance could be conferred via an alternative mechanism(s). Consequently, there is conflicting evidence in the literature reporting the correlation between phenotype and genotype (5, 34-37). The sensitivities and specificities reported ranged from 41% to 100% and 67% to 100%, respectively. This could be explained by the reliability of the reference standard which as mentioned above is highly susceptible to pH and inoculum size (14, 16, 17) and the critical concentration used. Alternatively, other mechanisms may be involved such as the gene *rpsA* (38). However, in a recent study reporting only a 45.7% correlation between genotype and phenotype also report no mutations could be identified in the *rpsA* gene (37). This suggests that *rpsA* may not be an alternative mechanism of PZA resistance. The authors cautioned against using genotypic results of *pncA* as the sole indicator that an isolate is resistant, and that DST should be used as the standard to define an isolate as PZA resistant.

In order for a rapid genetics diagnostic test to be developed, the correlation between genotype and phenotype must be better understood. A meta-analysis performed in 2012 investigated the role of different techniques by evaluating their test performance (39). The study found a very good correlation between genotype and phenotype. The authors suggest that direct DNA sequencing of the *pncA* gene would allow for a more rapid turnaround time and a lower cost incurred may be useful when compared to the BACTEC radiometric system (39). A study which investigated PZA resistance observed an excellent correlation between genotype and phenotype (5). However there are studies which have seen a much lower correlation with no explanation as to why this might be (37). This suggests that more work should be done in order to better understand the mode of action and other possible mechanisms of PZA resistance. This highlights the various techniques which are currently employed to assess PZA resistance. Due to the lack of a standardized technique the global distribution of PZA resistance has not been described.

Importance of Pyrazinamide

Even though there are still many unanswered questions with regards to PZA, it will continue to play an important role in anti-TB treatment therapy. This point is highlighted by the fact that PZA is

included in the latest WHO update on MDR-TB treatment guidelines (9). PZA is also included in many clinical trials where researchers are investigating novel possible antibiotic combinations such as the PaMZ trial (40). These two clinically relevant facts indicate that PZA will continue to be an important antibiotic in the fight against not only drug susceptible TB but drug resistant TB.

This thesis aims to further the current knowledge of PZA resistance in *M. tuberculosis* by addressing the following key knowledge gaps:

1. Regional and global estimates of PZA resistance
    - Hypothesis: PZA resistance is more prevalent both regionally and globally then currently known.
2. The prevalence of PZA resistance in different drug resistant cohorts (pan-susceptible to XDR-TB)
    - Hypothesis: The prevalence of PZA resistance increases as the more resistance is gained.
3. Mutations in the *pncA* gene which are not associated with PZA resistance
    - Hypothesis: Not all mutations in the *pncA* gene confer resistance at 100 µg/ml.
4. The effect of different mutations in the *pncA* gene on the organism
    - Hypothesis: Different mutations in the *pncA* gene affect the fitness of *M. tuberculosis*.
5. To investigate the possibility of a novel rapid genotypic diagnostic for PZA
    - Hypothesis: A rapid molecular diagnostic is possible using *pncA* as a marker for PZA resistance.

Structure of Thesis

Each chapter is structured for potential publication. If the results in a specific chapter have not been published yet, the references were formatted according to instructions of the Journal of Clinical Microbiology.

Chapter 1

The general introduction sets the tone for the thesis and introduces PZA and highlights the knowledge gaps and importance of this antibiotic.

Chapter 2

This published chapter serves to address a major knowledge gap, as it summarizes all literature of the prevalence of PZA resistance. The chapter provides regional estimates of PZA resistance as well as a global estimate. The review also addresses all mutations described in the literature that has been associated with PZA resistance.

Chapter 3

This published chapter comprises of a large data set of South African clinical isolates and illustrates the prevalence of PZA resistance from a pan-susceptible to XDR-TB; this chapter also demonstrates the correlation of genotypic to phenotypic data for predicting PZA resistance.

Chapter 4

This published chapter highlights mutations found in clinical isolates from both South Africa and the United States of America that were found to not confer resistance to PZA at the standard 100 µg/ml. This data is an important resource for researchers involved in designing novel rapid diagnostics for PZA.

Chapter 5

This chapter illustrates that different mutations in the *pncA* gene have an effect on the *in vitro* growth rate of the organism. While *pncA* is a non-essential gene, it is important to note that large deletions appear to affect the organism's ability to grow, and this may have an effect on the ability of the organism to transmit, however this does not appear to be the case.

Chapter 6

The chapter demonstrates a novel technique known as LATE-PCR coupled with Lights-On/Lights-Off probes to rapidly predict PZA resistance in a clinical isolate dataset. This technique shows highly promising results as a novel diagnostic.

Chapter 7

The general conclusion brings all the thesis chapters together and highlights how knowledge gaps described in the introduction have been addressed. This thesis has added significant value to the understanding of PZA resistance in *M. tuberculosis*.

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Chapter 2

A Global Perspective on Pyrazinamide Resistance: Systematic Review and Meta- Analysis

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My contribution:

- Research of data
- Literature search
- Data abstraction and interpretation
- Planning of manuscript
- Writing and editing of manuscript
- Addressed reviewer's comments

Abstract

Background

Pyrazinamide (PZA) is crucial for tuberculosis (TB) treatment, given its unique ability to eradicate persister bacilli. The worldwide burden of PZA resistance remains poorly described.

Methods

Systematic PubMed, Science Direct and Scopus searches for articles reporting phenotypic (liquid culture drug susceptibility testing or pyrazinamidase activity assays) and/or genotypic (polymerase chain reaction or DNA sequencing) PZA resistance. Global and regional summary estimates were obtained from random-effects meta-analysis, stratified by presence or risk of multidrug resistant TB (MDR-TB). Regional summary estimates were combined with regional WHO TB incidence estimates to determine the annual burden of PZA resistance. Information on single nucleotide polymorphisms (SNPs) in the *pncA* gene was aggregated to obtain a global summary.

Results

Pooled PZA resistance prevalence estimate was 16.2% (95% CI 11.2-21.2) among all TB cases, 41.3% (29.0-53.7) among patients at high MDR-TB risk, and 60.5% (52.3-68.6) among MDR-TB cases. The estimated global burden is 1.4 million new PZA resistant TB cases annually, about 270,000 in MDR-TB patients. Among 1,815 phenotypically resistant isolates, 608 unique SNPs occurred at 397 distinct positions throughout the *pncA* gene.

Interpretation

PZA resistance is ubiquitous, with an estimated one in six incident TB cases and more than half of all MDR-TB cases resistant to PZA globally. The diversity of SNPs across the *pncA* gene complicates the development of rapid molecular diagnostics. These findings caution against relying on PZA in current and future TB drug regimens, especially in MDR-TB patients.

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Introduction

The global burden of tuberculosis (TB) remains a major concern for health authorities worldwide. In 2013, there were an estimated 9.0 million new cases and 1.5 million deaths from TB [1]. Treatment regimens for drug-susceptible TB consist of rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB). PZA forms a critical cornerstone of this regimen given its unique ability to eradicate persister bacilli, which allowed treatment shortening from 9-12 months to 6 months [2,3]. PZA will likely remain an important component of treatment regimens for drug-susceptible and multidrug-resistant TB (MDR-TB) because of its distinctive mode of action (interference with ATP production) [4,5] and its synergistic pharmacokinetic properties with two of the new anti-TB drugs: the diarylquinoline Bedaquiline (affects F1F0 proton ATP synthase) and the nitroimidazole PA-824 (enhances PZA activity by altering the cell wall integrity) [6-8].

PZase is only active at low pH (pH 5.00-6.00) as experienced in the phagosomal compartment. Down-regulation of efflux pumps in the persister *Mycobacterium tuberculosis* results in intracellular accumulation of POA, which leads to the depletion of membrane potential [15-18], and inhibits trans-translation [5]. The decreasing membrane potential is detrimental to non-replicating persisters whose energy requirements are finely balanced [9]. Trans-translation plays a role for stress survival and pathogenesis as it aids the management of stalled ribosomes, damaged mRNA and proteins during stressful conditions [15,19-21].

Even though PZA is a crucial component of TB treatment, little is known about the prevalence of PZA resistance, particularly on a global scale. PZA drug susceptibility testing (DST) is technically challenging and rarely performed as part of routine care or routine drug surveillance in resource-limited settings. Two phenotypic PZA DSTs, BACTEC 460TB and BACTEC MGIT 960 (Becton Dickinson, Sparks, MD) exist; only BACTEC MGIT 960 is currently commercially available. Neither of these assays has been approved by the World Health Organization (WHO), likely due to their complexity and inconsistency, with frequent false positive results [22,23]. Classic and modified Wayne's PZase methods, which assess the function of the PZase enzyme based on a colorimetric change at critical concentrations of 100µg/ml to 400µg/ml, respectively [24], are also not endorsed by the WHO. More recently, genotypic PZA assays have been developed based on observations that

mutations in the *pncA* gene are the primary mechanism of PZA resistance [14,15,25-27]. The *pncA* gene encodes the pyrazinamidase (PZase) enzyme, which converts PZA, a pro-drug, into the active pyrazinoic acid (POA) [14,15]. These molecular techniques, albeit not approved by WHO, are the most commonly used techniques in PZA drug resistance studies.

Understanding regional differences in PZA resistance and its causal mutations is important for policy decisions regarding treatment regimens for drug-resistant TB and development of sequence-based diagnostics [26]. The aims of this review were to summarize the prevalence of PZA resistance globally and by WHO geographic regions, and to estimate the annual burden of PZA resistance, both stratified by MDR-TB status. We also summarize the global frequency and distribution of single nucleotide polymorphisms (SNPs) in the *pncA* gene in PZA resistant isolates.

Materials and Methods

Search strategy and selection criteria

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[28] We searched PubMed, ScienceDirect and Scopus on July 14, 2014 for relevant articles published in the English language between 1998 and 2014 using an *a priori* protocol. The search terms "tuberculosis AND (pyrazinamide OR PZA) AND (phenotype OR genotype OR PZase OR pyrazinamidase OR *pncA* OR BACTEC OR mutations OR resistance OR resistant OR susceptibility OR sequence analysis OR microbial sensitivity tests OR molecular typing)" were used to identify articles reporting on PZA resistance using any of the methods of interest: phenotypic PZA DST, PZase activity assays, and/or genotypic PZA assays. Additional articles were identified from reference lists and review articles.

Studies were eligible for inclusion in the meta-analysis of prevalence of PZA resistance if (1) PZA DST was assessed using at least one the following phenotypic tests: BACTEC liquid-based DST 460 or 960, considered the reference standard for PZA DST, or PZase activity assays using classic or modified Wayne's methods [24]. If both BACTEC 960 and BACTEC 460 results were reported, only the BACTEC 960 results were included. BACTEC 960 results were preferred to the BACTEC 460 due to the BACTEC 460 no longer being commercially available. If results from BACTEC 460 using

both a PZA concentration of 100µg/mL and 50µg/mL were reported, the results using 100µg/mL were included. If results from both classical and modified Wayne's PZase assays were reported, the classical Wayne's results were included. Authors were contacted if no clear method of PZA DST is described in the article. To be eligible for inclusion in the analysis, studies had to provide information on the MDR-TB risk status (patients diagnosed with MDR-TB, patients at high-risk of MDR-TB, or inclusion of any TB case), reporting on a single subgroup or stratifying results by subgroup. High-risk of MDR-TB was defined as an isolate being resistant to at least one anti-TB drug. Any TB was defined as the inclusion of patients independent of drug resistance profile.

Studies were eligible for inclusion in the descriptive SNP analysis if they performed genotypic testing using polymerase chain reaction (PCR) and DNA sequencing and characterized the found SNPs.

For both the PZA prevalence and *pncA* SNP analysis, studies including samples from multiple countries were only included if the results were stratified by country. In studies collecting multiple samples from a single patient, only the first sample result was used. Where a study performed repeat testing on a sample, only the first result was retained in the review. No additional exclusion criteria were imposed.

Data extraction

M. W. and R. W. independently reviewed titles and abstracts of original studies retrieved by the search. M. W. and T. Y. reviewed full-text and references of selected articles. M. W. and H.M.S. abstracted study data from full reports.

The following information, if available, was abstracted from each article: first author surname; publication year; WHO region (Africa, Americas, Eastern Mediterranean, Europe, South East Asia, or Western Pacific); study dates; study design; study setting; sample size; MDR risk subgroup; age; gender; HIV status; exclusion criteria; specimen type; phenotypic DST method; PZase activity assay; genotypic method; and whether the up and down stream regions of *pncA* were sequenced. The number of patients with PZA resistance according to liquid DST, the lack of PZase activity, or genotypic mutations in the *pncA* gene was also recorded. As Taiwan was not defined by the WHO, it was grouped in the Western Pacific region.

Statistical analysis

Summary estimates for the prevalence of PZA resistance, calculated using random-effects meta-analytic methods in STATA 13 (StataCorp LP, College Station, TX), were determined globally and for each WHO region, stratified according to whether the subgroup of patients had MDR-TB, were at high-risk of MDR-TB, or had any TB.

Estimation of the burden of PZA resistant TB, stratified by region and by presence of MDR-TB were obtained by multiplying the regional point estimates obtained by the random-effects meta-analysis by the most recent (2011) regional WHO estimates for incident TB and MDR-TB cases [1].

The analysis of SNPs in the *pncA* gene was descriptive. We present results according to nucleotide position in order to identify regions of SNP clustering within the *pncA* gene. In addition, we present a detailed description of all SNPs reported, including the location and type of polymorphism and countries where this SNP has been isolated, as well as whether this SNPs has been linked to a resistant phenotype only or has been observed in both resistant and susceptible isolates, with phenotypic resistance defined by BACTEC DST results or the PZase enzyme assay results if BACTEC DST result was not available.

Role of the funding source: The funders of the study (NIH and NRF) had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

Results

Selected studies

The literature search resulted in 1077 abstracts identified. Of these, 205 full-text articles were selected for review. In total, 62 [29-90] articles met the eligibility criteria for reporting PZA resistance, resulting in 91 datasets due to articles having isolates from multiple WHO regions as well as isolates which met different cohort type criteria (Fig. 1). Of the 205 full-text articles reviewed, 66 [27,59-123] articles were eligible for inclusion in the analysis of SNP frequency and distribution.

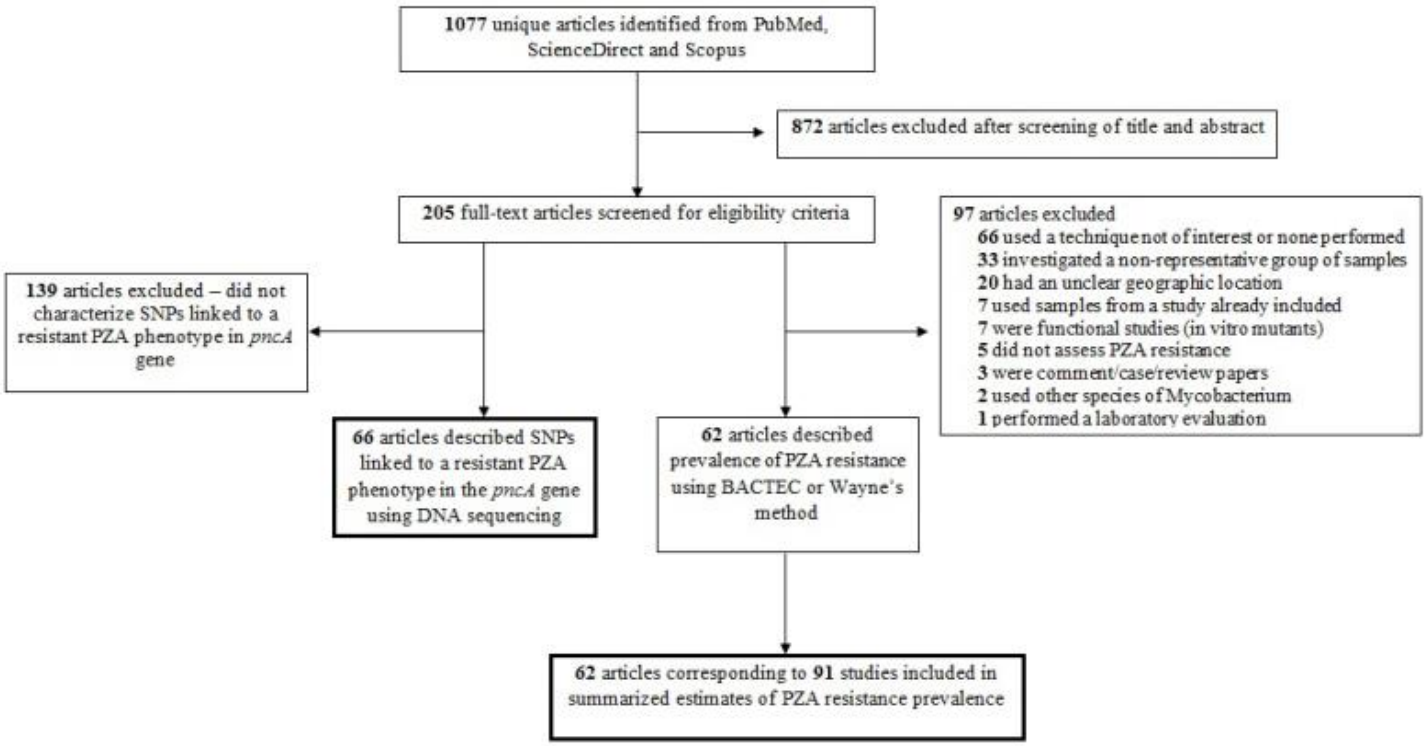

Figure. 1. Flow diagram describing article selection.

Phenotypic PZA Resistance: Study and Population Characteristics

The 62 [29-90] final studies provided phenotypic PZA resistance data on 35,950 *M. tuberculosis* clinical isolates. According to WHO regions, 8 [39,59,60,68,69,78] studies were from African region, 20 [29,30,38,40,41,43-47,59,70-72,79-83] from the Americas, 3 [31,48,80] from the Eastern Mediterranean, 20 [32,33,49-54,59,61,62,84-86] from European, 17 [34-36,47,55,56,59,63,73,80] from South East Asia, and 23 [37,42,57,58,64-67,74-77,80,86-90] from the Western Pacific region (Fig. 2). Most (53/91) [30,35,36,39-61,63,64,78-90] estimates of PZA prevalence were provided for studies including any TB patient, independent of drug resistance profile; 25 [29-37,42,59-67] studies reported PZA resistance among individuals with confirmed MDR-TB, and 13 [33,34,38,68-77] estimates were available for individuals at high-risk of MDR-TB. Study and population characteristics are displayed in S1 Table.

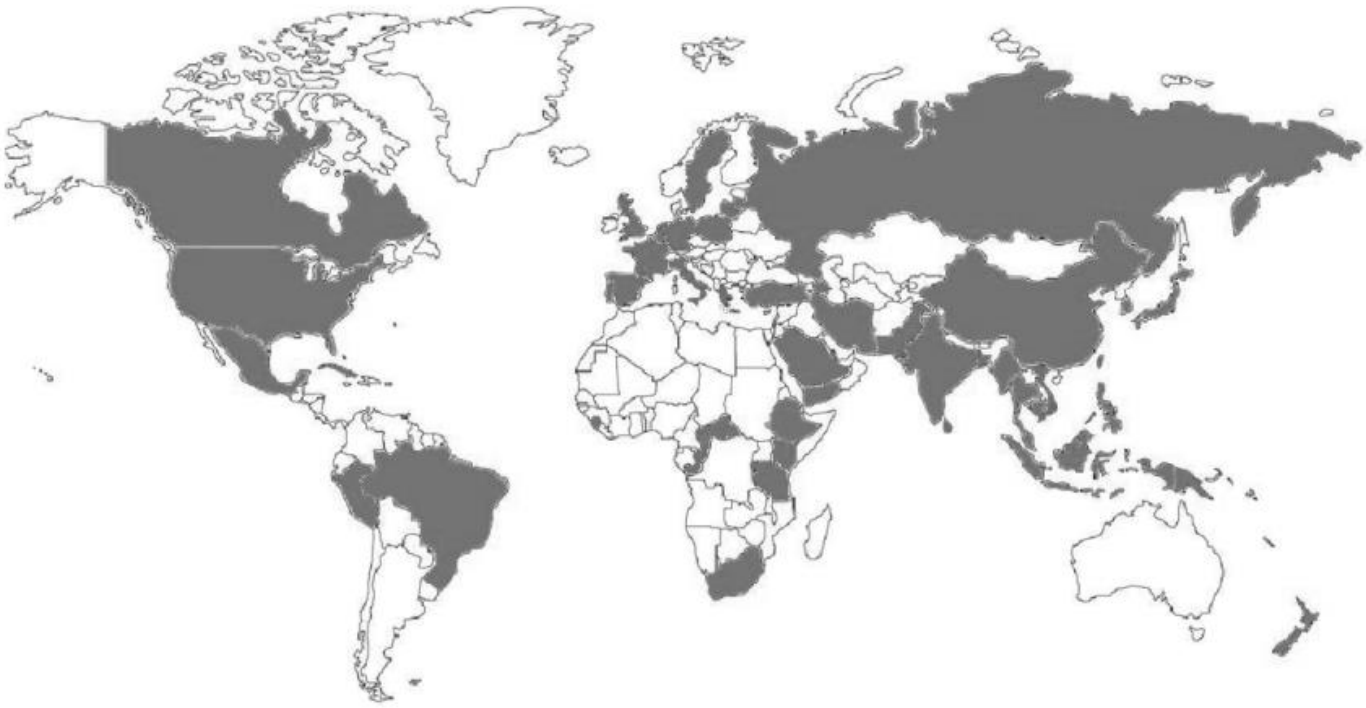

Figure. 2. Global distribution of included studies. Countries are shaded if a study was included in this review.

Phenotypic PZA Resistance: Regional and Pooled Prevalence's and Annual Burden

PZA resistance is prevalent across the entire globe and has been reported in all six WHO-defined regions (Fig. 3). The pooled summarized prevalence estimate of PZA resistance was 60.5% (95% CI 52.3-68.6%) in MDR-TB patients, 41.3% (95% CI 29.0-53.7%) in TB patients at high-risk of MDR-TB, and 16.2% (95% CI 11.2-21.2%) in studies including any TB patient irrespective of resistance profile. In all six WHO regions, the prevalence of PZA resistance was two to six times higher in MDR-TB patients compared to the population of all TB patients.

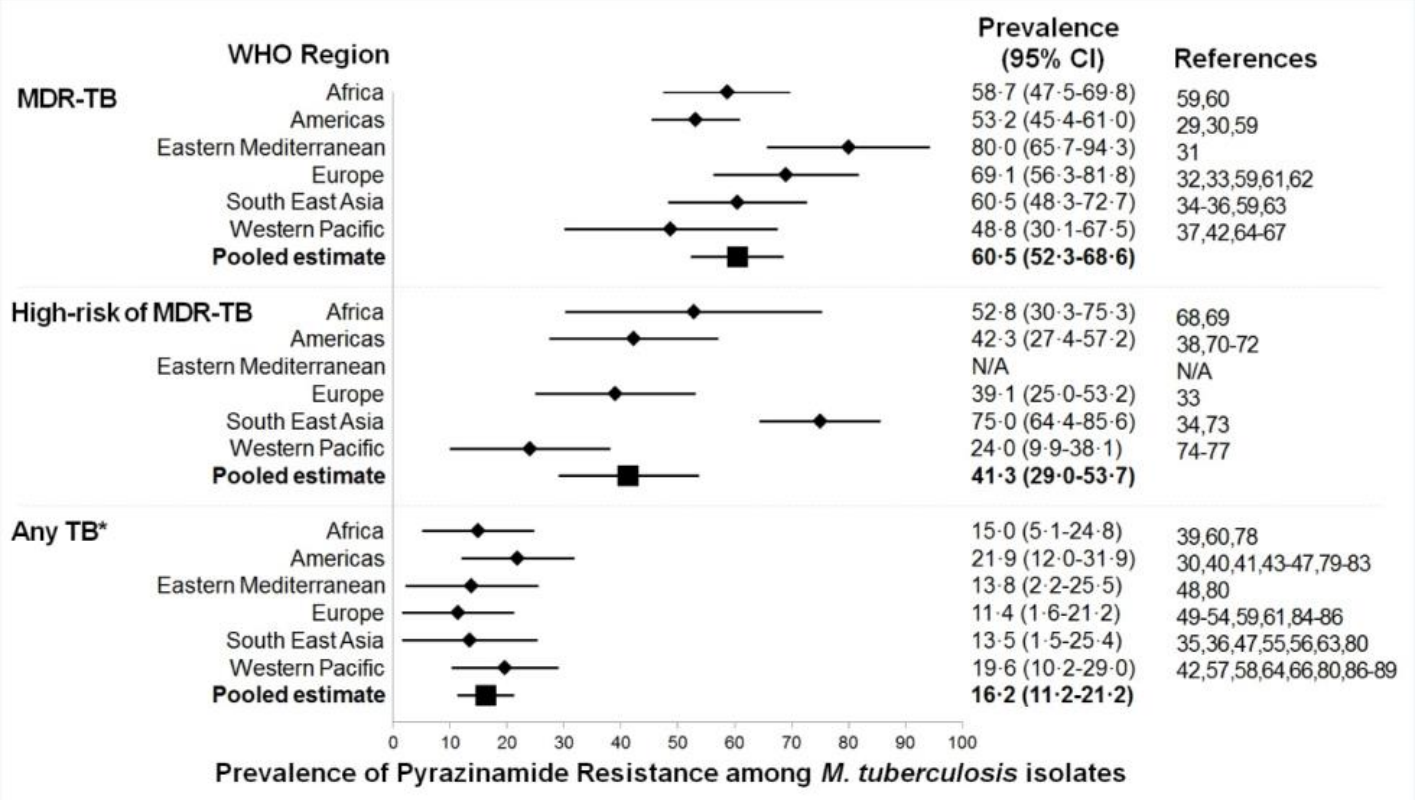

Figure 3. Forest plot for the summary estimates of pyrazinamide prevalence by WHO region and presence or risk of MDR-TB. Abbreviations: CI, confidence interval; DST, drug susceptibility test; MDR-TB, multi-drug resistant tuberculosis; N/A, not applicable; WHO, world health organization. MDR-TB was defined as an isolate being resistant to RIF and INH. High risk of MDR-TB was defined as an isolate being resistant to at least one anti-TB drug. *Any TB was defined as the inclusion of patients independent of drug resistance profile.

PZA resistance prevalence among cases of MDR-TB ranged from 48.8% (95% CI 30.1-67.5%) in the Western Pacific to 80.0% (95% CI 65.7-94.3%) in the Eastern Mediterranean, but the latter estimate was based on a single study [31]. The estimated PZA prevalence among those at high risk of MDR-TB varied greatly, from 24.0% (95% CI 9.9-38.1%) in the Western Pacific region to 75.0% (95% CI 64.4-85.6%) in South East Asian region. In the general TB population, PZA prevalence estimated

ranged from 11.4% (95% CI 1.6-21.2) in the European region to 21.9% (95% CI 12.0-31.9%) in the Americas.

Multiplying the regional WHO estimates for the annual number of new TB cases and incident MDR-TB cases by the pooled summarized prevalence estimates of PZA resistance, we estimated that about 1.4 million PZA resistant TB cases occur annually, corresponding to 16.2% of the 9.0 million incident TB cases in 2013 (Table 1). Of these, an estimated 270,000 occur in people also resistant to at least isoniazid and rifampicin, representing 60% of all incident cases of MDR-TB estimated in 2013.

Table 1. Estimated annual burden of new PZA resistant tuberculosis cases, overall and among MDR-TB patients, globally and by WHO region

WHO region	**Incident TB cases***	**Incident PZA resistant cases**	**Incident MDR-TB cases***	**Incident PZA resistant MDR-TB cases**
African	2,600 000	416,000	78,000	45,800
Americas	280,000	44,800	8,400	4,468
Eastern Mediterranean	750,000	120,000	27,000	21,600
European	360,000	57,600	91,000	62,881
South East Asian	3,400 000	544,000	135,000	81,675
Western Pacific	1,600 000	256,000	125,000	61,000
GLOBAL	9,000 000	1,438 000	464,400	277,424

Abbreviations: MDR-TB, multi-drug resistant tuberculosis; PZA, pyrazinamide; TB, tuberculosis; WHO, World Health Organization.

* Incidence of TB cases from the World Health Organization Global Tuberculosis Report 2014.

Single Nucleotide Polymorphism Distribution in *pncA* gene

The 66 [27,59-123] articles provided SNP data from 8,651 *M. tuberculosis* clinical isolates. According to WHO region, five [60,68,69,78,102] articles were from Africa, 14 [27,70-72,79,81-83,97,100,105,107,114,120] from the Americas, two [80,92] from the Eastern Mediterranean, 17 [61,62,84-86,93,96,103,104,106,108-110,115-117,119] from Europe, six [59,63,73,118,121,123] from South East Asia, and 22 [64-67,74-77,87-91,94,95,98,99,101,111-113,122] from the Western Pacific. A SNP in the *pncA* region was detected in the 1,815 of the 8,651 isolates, with 608 unique polymorphisms in 397 positions in the gene (S2 Table). SNPs were found throughout the entire *pncA* gene and flanking region with no particular clustering or hot spots (Fig. 4). There are however, a few SNPs which were found to be more frequently than others such as -11 and 195, but even the 20 most frequent SNPs only represented one third of all isolates with phenotypic PZA resistance.

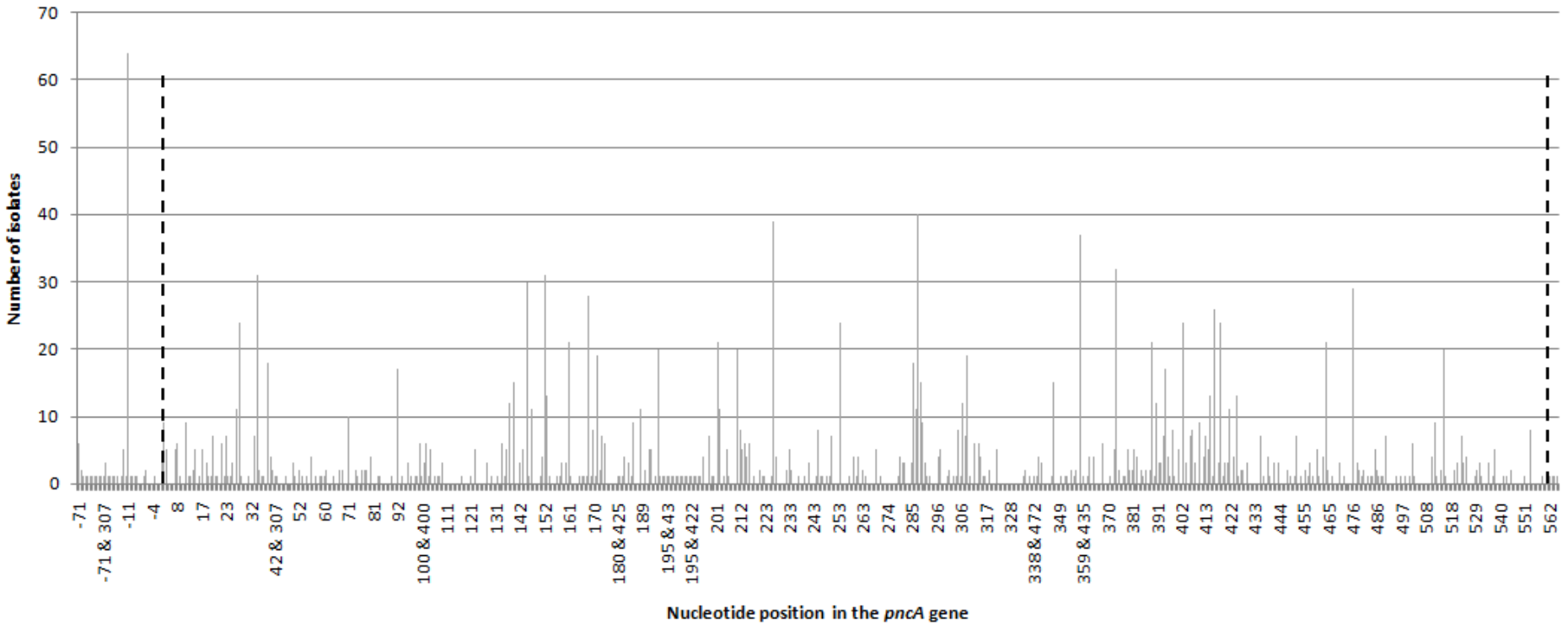

Figure 4. Distribution of reported single nucleotide polymorphisms (SNPs) throughout the *pncA* gene. Dashed lines indicate the open reading frame for the *pncA* gene.

Discussion

M. tuberculosis resistance to rifampicin and isoniazid is well described and monitored, either through continuous surveillance or periodic surveys of a representative sample of patients [124]. In contrast, resistance to ethambutol and PZA, the other two front line drugs, is not routinely monitored and thus poorly described. In this systematic review and meta-analysis, we found that PZA resistance is ubiquitous and increases in prevalence as risk of resistance to other drugs increases, with pooled summary estimates for the prevalence of PZA resistance of 16.2% in the total population of TB patients, 41.3% among TB patients at high risk of MDR-TB, and 60.5% in patients with confirmed MDR-TB. The high prevalence of PZA resistance results in an annual estimated burden of 1.4 million new cases of PZA resistant TB, of which about 270,000 occur in patients with MDR-TB [1]. This high prevalence of PZA resistance observed in all regions of the world and across different TB patient groups is an important finding as PZA is not only a key component of all current regimens for both drug susceptible and drug resistant TB but is also included in *all* novel drug regimens currently undergoing evaluation in clinical phase II or III trials for treatment of drug-susceptible or drug-resistant TB [125].

While our review aimed to comprehensively summarize information on PZA as a global public health problem, a different systematic review by Chang *et al* aimed to summarize the performance of molecular and PZase assays compared to culture-based phenotypic DST [126]. In that review, the median (range) of PZA resistance was 5% (0% to 9%) in non-MDR isolates and 51% (31% to 89%) in MDR *M. tuberculosis* isolates [126]. Our pooled prevalence estimates were higher than the median prevalence reported by Chang *et al*., especially in the overall TB patient population (16.2%). The summary estimate in our review may have overestimated the overall prevalence of PZA resistance among TB patients if the proportion of patients with drug resistance included in the studies was higher than that observed in the general population of the country where the studies took place.

The high prevalence of PZA resistance and its inclusion in both standard and novel drug regimens highlights the need for routine PZA resistance testing. Others have suggested that molecular assays may be the way forward for detecting PZA resistance, based on findings that molecular assays targeting *pncA* can detect PZA resistance in MDR-TB isolates with high positive predictive values

and rule out PZA resistance in non-MDR isolates with high negative predictive values [126,127]. However, DNA sequencing studies have revealed that mutations and/or polymorphisms occur across the entire length of the *pncA* gene, suggesting that sequencing the entire *pncA* gene would be essential to capture all possible mutations [109,117,127-129]. In this review, we confirmed that on a global scale, SNPs are distributed throughout the entire *pncA* gene. Whereas the tbdream database [15,26] previously reported on 278 unique polymorphisms prior to 2009, our systematic review provides an updated overview, with information on more than 600 unique polymorphisms in approximately 400 positions in *pncA* (including the upstream flanking region). A few SNPs occurred more frequently than others, possibly because these SNPs are rooted in ancestral strains. Consequently, developing a molecular assay to detect PZA resistance will be much more challenging compared to other genes (*rpoB, gyrA, embB*) which have been found to have clear resistance-causing hot spots [130]. The identification of causal PZA mutations is further complicated by the fact that not all non-synonymous mutations cause phenotypic resistance [80] and that mutations in the *pncA* gene can be absent in a small percentage of phenotypically PZA resistant isolates, [66,102] suggesting that PZA resistance could be conferred via an alternative mechanisms such as mutations in the *rpsA* gene [81]. Whereas development of simplified micro-array systems for simultaneous detection of rifampicin, isoniazid and ethambutol resistance may be possible, [131] inclusion of assessment of PZA resistance may thus require a different approach such as targeted DNA sequencing or next generation sequencing [127,132].

A major strength of our study was the comprehensive inclusion of studies from across the globe and stratification of estimates by region and TB patient category. Insight into PZA prevalence by region and TB patient category provides essential information for the development and clinical use of future PZA resistance tests. Our study adds to the review by Chang *et al*, which was aimed at summarizing PZA resistance assay performance, not PZA resistance prevalence. Our review also complements the recent study by Miotto *et al*, which presented *pncA* sequence results of 1950 clinical isolates obtained from multiple laboratories, but did not stratify results by regions or MDR-TB status [127]. Our review was however limited by the data quality of the original studies [126]. Misclassification of PZA resistance may have occurred in the studies included, and due to false positive results, may have

resulted in an overestimate of the true prevalence of PZA resistance. Phenotypic PZA drug susceptibility testing has not been endorsed by the WHO perhaps due to concerns surrounding false positivity related to the acidity of the media, inoculum size and critical concentration used [18,133,134]. Alternative techniques, the PZase activity assays, [24,135] have been used in the hope of identifying PZA resistance more accurately, but the interpretation of colourmetric change for these assays is highly subjective [56]. It is uncertain whether all mutations observed in the *pncA* region are associated with resistance. Similar to the tbdream database approach, [26] we chose not to make *a priori* decisions as to whether mutations described actually confer resistance and report any mutation found in a PZA-resistant isolate. Another limitation was the restricted number of studies for certain regions (especially Eastern Mediterranean, where all strains included came from a single study in Pakistan) and the lack of adequate representation of countries within certain regions (especially for Africa, where almost all isolates included came from South Africa, and the Americas). This not only resulted in uncertainty of the accuracy of the point estimates and wide confidence intervals but also highlights the lack of information on PZA resistance in several regions of the world. Finally, many studies did not provide clinical information and we were therefore unable to stratify our analysis by new versus re-treatment status.

The ubiquitous presence of PZA resistance is of global interest and should signal a call to action. Development of rapid diagnostics to detect PZA resistance will be essential to maximize the efficacy of novel treatment regimens and minimize the risk of development of resistance to novel drugs. In addition, the high prevalence of PZA resistance, especially among MDR-TB patients, highlights the need for development of treatment regimens that can be effectively used in patients with PZA-resistant MDR-TB.

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Conflicts of interest

None reported.

Author contributions

Concept and design: M. W., R. W., A. V. R. Literature search: M. W., T. Y. Figures and Tables: M. W., T. Y., H. M. S., A. V. R., R. W. Data abstraction: M. W., H. M. S., T. Y. Drafting of the article: M. W., H. M. S. Data analysis: H. M. S., A. V. R. Data interpretation: M. W., H. M. S., E. M. S., A. V. R., R. W. Critical revision for important intellectual content and final approval of article: M. W., H. M. S., E. M. S., S. S., P. V. H., A. V. R., R. W.

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Supporting Information

S1 Table. Additional study and population characteristics. Abbreviations: MDR-TB, multi-drug resistant tuberculosis; PZA, pyrazinamide; TB, tuberculosis; WHO, World Health Organization; lab, laboratory; N/A, not applicable; N/S, not stated; HR-MDR, high-risk multi-drug resistant tuberculosis; MTB, *Mycobacterium tuberculosis;* PCR, polymerase chain reaction, East Med, Eastern Mediterranean

S2 Table. Single-nucleotide polymorphisms (SNPs) detected in the *pncA* gene, by country and resistance phenotype. Abbreviations: A, adenine; bp, base pair; C, cytosine; del, deletion; G, guanine; R, resistant; SNP, single-nucleotide polymorphism; T, thymine.

* Article found one isolate sensitive and one isolate resistant.

Supplemental Table 1. Additional study and population characteristics.

WHO Region	Country	Study Design	Study Setting	Cohort Type	Sample Size	Specimen Type	Phenotypic Method	PZase Activity Assay	Genotypic Method	Reference
Africa	**Central African Republic**	Retrospective	Lab	N/A	54	Sputum	N/A	No	PCR and DNA sequencing	Minime-Lingoupou 2010
	Congo	Retrospective	Lab	MDR	1	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Mestagh 1999
	Kenya	Prospective	Clinical	Any TB	286	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	N/A	Ndung'u 2012
	Rwanda	Retrospective	Lab	MDR	1	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Mestagh 1999
	Tanzania	Retrospective	Lab	N/A	13	MTB clinical isolates	N/A	No	PCR and DNA sequencing	Mpagama 2013
	South Africa	N/S	N/S	HR-MDR	27	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Davies 2000
		Retrospective	Lab	N/A	9	MTB clinical isolates	N/A	No	Whole genome sequencing	Ioerger 2009
		Retrospective	Lab	Any TB	26	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	Ion Torrent	Daum 2012
		Retrospective	Lab	N/A	21	MTB clinical isolates	BACTEC 460 (100-900µg/ml)	Yes	PCR and DNA sequencing	Bishop 2001
		Retrospective	Clinical	HR-MDR	174	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Louw 2006
		Retrospective	Lab	MDR; any TB	221	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Mphahlele 2008
Americas	**Brazil**	Retrospective	Lab	N/A	40	MTB clinical isolates	Indirect proportion method	Yes	PCR and DNA sequencing	Rodrigues 2005
		Retrospective	Lab	N/A	36	MTB clinical isolates	Proportion method	Yes	PCR and DNA sequencing	Barco 2006
		Prospective	Clinical	HR-MDR	23	Sputum	BACTEC 460 (N/S)	Yes	PCR and DNA sequencing	Clemente 2008
		Prospective	Clinical	Any TB	71	MTB clinical isolates	7H9 agar	Yes	N/A	Ribeiro 2012
		Retrospective	Clinical	Any TB	97	MTB clinical isolates	Proportion method	Yes	PCR and DNA sequencing	Bhuju 2013

		Prospective	Lab	Any TB	50	Sputum	BACTEC 460 (100µg/ml)	No	N/A	Barreto 2002
		Retrospective	Lab	N/A	32	MTB clinical isolates	N/A	Yes	PCR and DNA sequencing	Ghiraldi 2011
	Canada	Retrospective	Lab	Any TB	28	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Hirano 1998
		Retrospective	Lab	N/A	28	MTB clinical isolates	BACTEC 460 (100-300µg/ml)	Yes	PCR and DNA sequencing	Cheng 2000
		Prospective	Lab	Any TB	743	MTB clinical isolates	BACTEC 460/960 (100µg/ml)	Yes	PCR and DNA sequencing	Chedore 2010
		Retrospective	Lab	Any TB	141	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	PCR and DNA sequencing	Alexander 2012
		Retrospective	Lab	MDR	1	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Mestagh 1999
		Retrospective	Lab	N/A	354	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and RFLP	Nguyen 2003
	Cuba	Retrospective	Lab	Any TB	102	MTB clinical isolates	Nitrate reductase assay	Yes	PCR and DNA sequencing	Mirabal 2010
	Mexico	Prospective	Lab	HR-MDR	127	Sputum	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Cuevas-Cordoba 2013
		Prospective	Lab	Any TB	460	MTB clinical isolates	BACTEC 460 (N/S)	No	N/A	Granich 2000
	Peru	Prospective	Clinical	HR-MDR	29	MTB clinical isolates	N/A	Yes	PCR and DNA sequencing	Escalante 1998
		Retrospective	Lab	Any TB	185	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Sheen 2009
		Prospective	Clinical	MDR	150	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	N/A	Becerra 2000
		Retrospective	Clinical	HR-MDR	122	MTB clinical isolates	N/A	Yes	N/A	Saravia 2005
	United	Retrospective	Lab	Any TB	16	MTB clinical isolates	BACTEC 460 (N/S)	No	PCR and DNA sequencing	Sachais 1998
	States	Retrospective	Lab	MDR; Any TB	48	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	N/A	LaBombardi 2002
		Retrospective	Lab	N/A	19	MTB clinical isolates	BACTEC 460 (100-900µg/ml)	Yes	PCR and DNA sequencing	Cheng 2000
		Retrospective	Lab	N/A	60	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Morlock 2000
		Retrospective	Lab	N/A	1436	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	PCR and DNA sequencing	Hannan 2001
		Retrospective	Lab	N/A	33	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Denkin 2005
		Retrospective	Lab	Any TB	54	MTB clinical isolates	BACTEC 460/960 (100µg/ml)	No	N/A	Fredriks 2006
		Retrospective	Lab	N/A	29	MTB clinical isolates	BACTEC 460 (6.25-50µg/ml)	No	PCR and DNA sequencing	Dormandy 2007
		Prospective	Lab	Any TB	27	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	N/A	Duque 2013

East Med.	**Iran**	Prospective	Clinical	N/A	49	MTB clinical isolates	Middlebrook 7H9	Yes	PCR and DNA sequencing	Doustdar 2009
	Yemen	Retrospective	Lab	Any TB	31	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Hirano 1998
	Pakistan	Prospective	Clinical	MDR	30	MTB clinical isolates	BACTEC 960 (50-100µg/ml)	No	N/A	Khurram 2012
	Saudi Arabia	Prospective	Lab	Any TB	101	MTB clinical isolates	BACTEC (N/S)	No	N/A	Khan 2001
Europe	**Abkhazia**	Prospective	Clinical	MDR	37	Sputum	BACTEC 960 (100µg/ml)	No	N/A	Pardini 2007
	Azerbaijan	Retrospective	Lab	MDR	5	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Mestagh 1999
	Belgium	Retrospective	Lab	N/A	138	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Stoffels 2012
		Retrospective	Lab	MDR	2	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Mestagh 1999
	France	Retrospective	Lab	Any TB	35	MTB clinical isolates	Middlebrook 7H10	Yes	PCR and DNA sequencing	Lemaitre 1999
	Germany	Retrospective	Lab	N/A	9	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	PCR and DNA sequencing	Napiorkowska 2014
	Greece	Prospective	Lab	Any TB	150	MTB clinical isolates	BACTEC 460/960 (100µg/ml)	No	N/A	Kontos 2003
	Italy	Retrospective	Lab	Any TB	337	MTB clinical isolates	BACTEC 460 (N/S)	No	N/A	Nutuni 1998
		Retrospective	Lab	MDR; HR-MDR	46	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	N/A	Fattorini 1999
		Retrospective	Lab	Any TB	100	MTB clinical isolates	BACTEC 460/960 (100µg/ml)	No	N/A	Scarparo 2004
		N/S	N/S	MDR; Any TB	201	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Piersimoni 2013
	Latvia	Retrospective	Clinical	N/A	28	MTB clinical isolates	BACTEC 460 (N/S)	No	PCR and DNA sequencing	Tracevska 2004
	Netherlands	Retrospective	Lab	Any TB	1831	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Simons 2012
	Poland	Retrospective	Lab	Any TB	33	MTB clinical isolates	BACTEC 960 (100µg/ml)	Yes	PCR and DNA sequencing	Sekiguchi 2007
		Retrospective	Lab	Any TB	1909	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	N/A	Napiorkowska 2010
		Retrospective	Lab	N/A	72	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	PCR and DNA sequencing	Napiorkowska 2014
	Portugal	Retrospective	Clinical	N/A	55	MTB clinical isolates	BACTEC 960 (100µg/ml)	Yes	PCR and DNA sequencing	Portugal 2004
		Retrospective	Lab	MDR	58	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Perdigao 2008
	Russia	Prospective	Clinical	N/A	44	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Marttila 1999

		Prospective	Prison	N/A	75	MTB clinical isolates	N/A	No	PCR and DNA sequencing	Shemyakin 2004
		Prospective	Clinical	N/A	21	MTB clinical isolates	N/A	No	PCR and DNA sequencing	Zhdanova 2013
		Retrospective	Lab	MDR	3	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Mestagh 1999
	Spain	Retrospective	Lab	N/A	50	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	PCR and DNA sequencing	Aragon 2007
		Retrospective	Clinical	Any TB	57	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	PCR and DNA sequencing	Espasa 2012
	Scotland	Retrospective	Lab	Any TB	6	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Mestagh 1999
	Sweden	Retrospective	Lab	N/A	69	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	PCR and DNA sequencing	Jureen 2008
		Prospective	Lab	N/A	52	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Hoffner 2013
		Retrospective	Clinical	N/A	46	MTB clinical isolates	BACTEC 460/960 (100µg/ml)	Yes	PCR and DNA sequencing	Werngren 2012
	Turkey	Retrospective	Lab	N/A	10	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Brown 2000
		Retrospective	Lab	N/A	12	MTB clinical isolates	BACTEC 460 (N/S)	Yes	PCR and DNA sequencing	Yuksel 2009
	United Kingdom	Retrospective	Lab	Any TB	20899	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	N/A	Djuretic 2002
South East Asia	**Bangladesh**	Retrospective	Lab	MDR	4	MTB clinical isolates	BACTEC 460 (100µg/ml)	Yes	PCR and DNA sequencing	Mestagh 1999
	India	Retrospective	Lab	Any TB	10	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Hirano 1998
		Prospective	Clinical	N/A	65	Sputum	N/A	No	PCR and DNA sequencing	Stavrum 2009
		Retrospective	Clinical	N/A	77	MTB clinical isolates	N/S	No	PCR and DNA sequencing	Harris 2000
		Prospective	Clinical	HR-MDR	50	Sputum	Proportion method	Yes	PCR and DNA sequencing	Muthaiah 2010
		N/S	Clinical	Any TB	130	MTB clinical isolates	BACTEC 960 (100µg/ml)	Yes	N/A	Krishnamurthy 2004
		Retrospective	Lab	N/A	107	MTB clinical isolates	Proportion method	Yes	N/A	Singh 2007
		Prospective	Clinical	N/A	63	MTB clinical isolates	BACTEC 960 (100µg/ml)	Yes	PCR and DNA sequencing	Shenai 2009
		Retrospective	Lab	Any TB	136	MTB clinical isolates	BACTEC 960 (100µg/ml)	Yes	N/A	Sharma 2010
		Retrospective	Lab	Any TB	186	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	N/A	Arora 2013
	East Timor	Prospective	Clinical	MDR; HR-MDR	23	MTB clinical isolates	N/A	Yes	N/A	Kelly 2004

	Indonesia	Retrospective	Lab	Any TB	8	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Hirano 1998
		Prospective	Clinical	Any TB	103	MTB clinical isolates	N/A	Yes	N/A	Kelly 2006
	Myanmar	Retrospective	Lab	Any TB	16	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Hirano 1998
	Thailand	Retrospective	Lab	Any TB	54	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Hirano 1998
		Prospective	Lab	Any TB	30	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	N/A	Duque 2013
		Retrospective	Lab	N/A	42	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Watcharasamphankul 2013
		Retrospective	Lab	N/A	98	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Pholwat 2014
		Retrospective	Lab	MDR; Any TB	150	MTB clinical isolates	BACTEC 960 (100µg/ml)	Yes	PCR and DNA sequencing	Jonmalung 2010
Western Pacific	**Cambodia**	Retrospective	Lab	N/A	166	MTB clinical isolates	N/A	No	PCR and DNA sequencing	Pierre-Audigier 2012
	China	Retrospective	Lab	N/A	64	MTB clinical isolates	BACTEC 460 (100µg/ml)	No	PCR and DNA sequencing	Hou 2000
		Retrospective	Lab	N/A	47	MTB clinical isolates	Proportion method	Yes	PCR and DNA sequencing	Zhang 2009
		Prospective	Clinical	Any TB	51	MTB clinical isolates	BACTEC 960 (100µg/ml)	Yes	PCR and DNA sequencing	Zhou 2011
		Prospective	Clinical	N/A	53	Sputum	N/A	Yes	Long fragment qPCR	Li 2014
		Prospective	Clinical	Any TB	432	MTB clinical isolates	BACTEC 960 (100µg/ml)	Yes	PCR and DNA sequencing	Cui 2013
		Retrospective	Clinical	Any TB	132	MTB clinical isolates	Proportion method	Yes	PCR and DNA sequencing	Huang 2013
		Prospective	Clinical	MDR; Any TB	218	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	PCR and DNA sequencing	Tan 2013
	Malaysia	Retrospective	Lab	Any TB	1	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Hirano 1998
	New Zealand	Prospective	Lab	N/A	33	MTB clinical isolates	BACTEC 460/960 (100µg/ml)	Yes	PCR and DNA sequencing	Pandey 2009
	Philippines	Retrospective	Lab	Any TB	12	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Hirano 1998
	Papua New Guinea	Retrospective	Clinical	MDR	39	MTB clinical isolates	BACTEC 460/960 (100µg/ml)	No	N/A	Simpson 2011
	Korea	Retrospective	Lab	Any TB	8	MTB clinical isolates	Middlebrook 7H11	Yes	PCR and DNA sequencing	Hirano 1998
		Retrospective	Lab	N/A	10	MTB clinical isolates	Proportion method	Yes	PCR and DNA sequencing	Cheng 2000

		Retrospective	Lab	N/A	95	MTB clinical isolates	N/A	Yes	PCR and DNA sequencing	Lee 2001
		Retrospective	Clinical	HR-MDR	93	Sputum	N/A	Yes	PCR and DNA sequencing	Choi 2010
		Retrospective	Lab	N/A	80	MTB clinical isolates	N/A	Yes	PCR and DNA sequencing	Yoon 2012
		Prospective	Clinical	N/A	102	MTB clinical isolates	Proportion method	Yes	PCR and DNA sequencing	Kim 2012
		Retrospective	Clinical	N/A	23	Extrapulmonary MTB	N/A	No	PCR and DNA sequencing	Lee 2012
		Retrospective	Lab	N/A	192	MTB clinical isolates	Absolute concentration method	No	PCR and DNA sequencing	Jnawali 2013
	Taiwan	Retrospective	Clinical	MDR; Any TB	76	MTB clinical isolates	BACTEC 960 (100-300µg/ml)	Yes	PCR and DNA sequencing	Huang 2003
		Retrospective	Clinical	Any TB	611	MTB clinical isolates	N/A	Yes	N/A	Su 2008
		Prospective	Clinical	MDR	66	MTB clinical isolates	BACTEC 960 (100µg/ml)	Yes	PCR and DNA sequencing	Chiu 2011
		Prospective	Lab	MDR; Any TB	56	MTB clinical isolates	BACTEC 960 (100µg/ml)	No	N/A	Huang 2013
	Vietnam	Retrospective	Clinical	Any TB	339	MTB clinical isolates	N/A	Yes	Line probe	Van Hung 2013

Abbreviations: MDR-TB, multi-drug resistant tuberculosis; PZA, pyrazinamide; TB, tuberculosis; WHO, World Health Organization; lab, laboratory; N/A, not applicable; N/S, not stated; HR-MDR, high-risk multi-drug resistant tuberculosis; MTB, *Mycobacterium tuberculosis;* PCR, polymerase chain reaction, East Med, Eastern Mediterranean.

Supplemental Table 2. Single-nucleotide polymorphisms (SNPs) detected in the *pncA* gene, by country and resistance phenotype

SNP location	SNP type	Country and reference	Phenotype linked to SNP	SNP Frequency
-71	1-bp deletion	Korea[1]	R	5
	2-bp deletion	Korea[1]	R	1
-71 & -11	1-bp deletion & A>G	Korea[1]	R	2
-71 & 20	1-bp deletion & T>G	Korea[1]	R	1
-71 & 56 & 386	1-bp deletion & A insertion & 10-bp insertion	Korea[1]	R	1
-71 & 58 & 400	1-bp deletion & G>A & G>A	Korea[1]	R	1
-71 & 67	1-bp deletion & C insertion	Korea[1]	R	1
-71 & 152	1-bp deletion & A>C	Korea[1]	R	1
-71 & 161	2-bp deletion & C>T	Korea[1]	R	1
-71 & 199	1-bp deletion & T>C	Korea[1]	R	1
-71 & 226	1-bp deletion & A>C	Korea[1]	R	1
-71 & 298	1-bp deletion & A>C	Korea[1]	R	1
-71 & 307	1-bp deletion & T>C	Korea[1]	R	1
-71 & 403	1-bp deletion & A>C	Korea[1]	R	3
-71 & 446 & 501	1-bp deletion & T insertion and T insertion	Korea[1]	R	1
-71 & 454	1-bp deletion & 10-bp deletion	Korea[1]	R	1
-71 & 478	1-bp deletion & A>C	Korea[1]	R	1
-16	AACGTA>GGCAGTT	USA[33]	R	1
-15 & 11	A>C & T>C	Sweden[57]	R	1
-13	G>T	Canada[64]	R	1
-12	T>C	South Africa[3], Netherlands[41]	R[3,41]	3
	T>G	Russia[4]	R	1
	T deletion	Iran[2]	R	1

-12 & 228	T>C & T>C	Korea[5]	R	1
-11	80-bp deletion	Korea[1]	R	1
-11 & 376	80-bp deletion & G>A	Korea[1]	R	1
-11	A>G	Korea[1,5,6,7], Iran[2], Russia[4], Thailand[8], Portugal[9], Scotland[10], Brazil[49], Canada[12,64], Peru[13], China[31,47], Sweden[38], India[50], Spain[59], Poland[66]	R[1,2,4,5,6,7,8,9,10,12,13,31,38,47,49,50,59,64,66]	62
-11 & 483	A>G & G>A	India[50]	R	1
-11 & 535	A>G & A deletion	India[50]	R	1
-11	A>C	Thailand[8]	R	1
-10	T>C	South Africa[3]	R	1
-7	T>C	USA[33]	R	1
-7 & 340	T>C & A>G	Japan[14]	R	2
-3	C insertion	Sweden[57]	*R/S	1
1	561-bp deletion	Japan[46]	R	1
	11-bp deletion	China[15]	R	1
	8-bp deletion	China[15]	R	1
2	T>C	Portugal[9,35], Poland[66]	R[9,35,66]	9
3	G>A	Japan[16], Netherlands[41]	R[16,41]	4
	G>T	Korea[6]	R	1
7	G>C	Iran[2], Brazil[11,17], France[37]	R[2,11,17,37]	4
	G>T	Taiwan[18]	R	1
8	C>A	Korea[1, 20], Japan[14,19,46]	R[1,14,19,20,46]	6
8 & 419	C>A & G>A	Japan[14]	R	1
11	T>C	Korea[1], Peru[32], USA[33], China[34,47], Japan[36]	R[1,32,33,34,36,47]	7
	T>G	Korea[1,6]	R[1,6]	2
12	G deletion	Cuba[56]	R	1
12 & 421	T insertion & C>T	Korea[1]	R	1
13	AT insertion	India[58]	R	2
14	T>A	Japan[16,46]	R[16,46]	2
	T>G	Japan[16], India[50]	R[16,50]	3
16	T>C	South Africa[21]	R	1
17	T>G	Korea[20], China[34]	R[20,34]	2
	T>C	Sweden[38,57,61]	R[38,57,61]	3
19	G>A	Peru[13]	R	1

	G>T	Korea[6], Peru[13]	R[6,13]	2
19 & 22	G>C & G>T	Korea[1]	R	1
19 & 403	G>C & A>C	Korea[1]	R	1
20	T>G	Russia[4], Yemen[22], Brazil[23], USA[39]	R[4,22,23,39]	5
	T>A	Korea[6]	R	1
	T>C	Brazil[11]	R	1
20 & 386 & 517	C insertion & 10-bp insertion & G deletion	Korea[1]	R	1
20 & 437	C insertion & C>T	Korea[1]	R	1
22	G>T	Russia[4], Belgium[43]	R[4,43]	2
	G>A	South Africa[3], China[34], Belgium[43]	R[3,34,43]	4
22 & 104	G>T & T>G	South Africa[3]	R	1
23	A>G	Korea[6,20], Spain[59], Canada[64]	R[6,20,59,64]	5
	A>C	China[31]	R	1
	TCG insertion	Korea[7]	R	1
24	C>G	Japan[46]	R	1
25	G>T	South Africa[24]	R	1
26	T>G	Korea[6], Japan[14]	R[6,14]	2
	T>C	Korea[6]	R	1
28	C>T	Russia[4], Korea[6,20], Brazil[11], Latvia[25], Belgium[43]	R[4,6,11,20,25,43]	7
	C>A	Russia[4], Spain[59]	R[4,59]	2
	C deletion	China[15]	R	2
29	A>C	Korea[1,6,20], Myanmar[22], China[31,48], USA[33,39], Japan[36], Belgium[43], Spain[59], Singapore[62], Poland [19,66]	R[1,6,19,20,22,31,33,36,39,43,48,59,62,66]	17
	A>G	Korea[6], USA[12], Peru[32], Cuba[56], Poland[66]	R[6,12,32,56,66]	7
29 & 295	A>C & T>G	Korea[6]	R	1
31 & 34 & 290	C insertion & G>T & G>A	Korea[1]	R	1
34	G>A	Korea[5], USA[39], Italy[40], China[63]	R[5,39,40,63]	7
35	A>C	Korea[6,20] ,USA[12], China[15,47], Japan[19], India[58], Mexico[26], Peru[32], Canada[64]	R[6,12,15,19,20,26,32,47,58,64]	20

	A>G	South Africa[3]	*R/S	9
	G insertion	South Africa[3]	R	2
36	A>G	Peru[32]	R	2
37	T>C	China[31]	R	1
38	T>C	France[37]	R	1
40	T>C	Korea[1,5,6], South Africa[3,24,60], Myanmar[22], Japan[46], China[47]	R[1,3,5,6,22,24,46,47,60]	14
	T>G	Peru[32]	R	3
	T deletion	South Africa[3]	R	1
41	G>A	Korea[6,7], Latvia[25], China[34]	R[6,7,25,34]	4
42	C>G	Korea[20]	R	1
	C>A	Poland[66]	R	1
42 & 307	C>G & T>C	Latvia[25]	R	1
42 & 535	C>A & A deletion	India[50]	R	1
46	G>A	China[47]	R	1
49	G>A	China[34], Singapore[62]	R[34,62]	2
	1-bp deletion	Korea[20]	R	1
50	G>A	Thailand[22]	R	1
52	T>C	New Zealand[45]	R	1
	G insertion	Canada[12]	R	1
52 &161	T>C & C>T	China[31]	R	1
54	2-bp insertion	Japan[16]	R	1
56	T>C	China[15], New Zealand[45]	R[15,45]	2
	T>G	Thailand[8]	R	1
	234-bp deletion	Korea[7]	R	1
57 & 62 & 70	3-bp deletion & 4-bp deletion & 5-bp deletion	China[48]	R	1
59	1-bp deletion	China[15]	R	1
59 & 232	1-bp deletion & G>A	Japan[14]	R	1
60	G deletion	Netherlands[41]	R	1
61	G insertion	Italy[40], China[47]	R[40,47]	2
64	A deletion	Thailand[65]	R	1
67	1-bp deletion	Korea[20]	R	1
	C insertion	Korea[1]	R	1
68	G>C	Taiwan[18]	R	1

68-69	GT>TC	Korea[6]	R	1
71	G>A	Turkey[27], China[31], Peru[32]	R[27,31,32]	5
	G deletion	China[15], USA[33,39]	R[15,33,39]	5
74	1-bp deletion	Korea[20]	R	1
	5-bp deletion	Brazil[17]	R	1
74-75 & 77 & 83	CC>AA & C>G & C>A	Korea[6]	R	1
76	GC insertion	Brazil[17]	R	2
77	5-bp insertion	Korea[1]	R	1
	G deletion	Korea[6]	R	1
78	G>C	India[58]	R	2
80	T>C	Thailand[8], China[47], India[50]	R[8,47,50]	4
83	C>T	Taiwan[18]	R	1
84	C deletion	Russia[4]	R	1
89	C>A	China[51]	R	1
92	T>C	Thailand[8]	*R/S	13
	T>G	Thailand[8]	*R/S	4
94	T>G	South Africa[24]	R	1
96 & 338	C>T & G>T	Russia[42]	R	3
96 & 338 & 362	C>T & G>T & G>T	Russia[42]	R	1
98	A>C	China[34]	R	1
99	C deletion	Sweden[38]	R	1
100	T>G	Japan[14,16,46], Peru[32]	R[14,16,32,46]	6
100 & 400	T>G & G>T	Japan[14]	R	1
101	A>G	Iran[2]	R	1
	A>C	Thailand[22]	R	2
102	C>A	Korea[5], Japan[14]	R[5,14]	2
	C>G	Netherlands[41]	R	1
	167-bp deletion	Turkey[27]	R	3
103	C deletion	Spain[59]	R	1
104	T>C	Thailand[8], Japan[46], Spain[59]	R[8,46,59]	4
	T>G	Brazil[23]	R	1
106	~5.3 kb deletion	USA[33]	R	1
107	CG insertion	South Africa[21]	R	1

108	C>G	China[47]	R	1
109	G>T	South Africa[24]	R	3
117 & 169	G>A & C>G	Sweden[38]	R	1
121	T>C	China[15]	R	1
123	C>G	China[15]	R	2
	C>A	Thailand[22], Peru[32]	R[22,32]	3
128	A>C	Thailand[22], Belgium[43]	R[22,43]	3
129 & 493	2-bp deletion & G>A	Japan[14]	R	1
132	A insertion	Korea[1]	R	1
134	T>A	Korea[1]	R	2
	T>G	Korea[6], Taiwan[18]	R[6,18]	2
	T>C	Thailand[8], China[34]	R[8,34]	2
135	G>C	Taiwan[18]	R	1
136	G>C	South Africa[24], Spain[59]	R[24,59]	2
	G>T	Thailand[8]	R	1
	G insertion	Yemen[22]	R	1
	G deletion	Brazil[17]	R	1
137	C>A	Korea[1,6,12]	R[1,6,12]	3
	C>T	Portugal[9], Canada[12,64], USA[12], Korea[12], Yemen[22]	R[9,12,22,64]	9
139	A>C	China[15], Korea[20]	R[15,20]	2
	A>G	Korea[6], Mexico[26], China[31], USA[33,39], Sweden[38]	R[6,26,31,38,39], *R/S[33]	12
	A>T	Russia[4]	R	1
142	A>G	Korea[1], China[47]	R[1,47]	3
143	A>C	Peru[32], Cuba[56]	R[32,56]	5
145	G>A	Korea[20], Peru[32]	R[20,32]	28
	G>C	South Africa[24]	R	1
	G>T	China[48]	R	1
145 & 305	G>C & C>T	Mexico[26]	R	1
146	A>T	Russia[4]	R	1
	A>G	Russia[4], Korea[20]	R[4,20]	2
	A>C	Korea[6,20], Brazil[23], Belgium[43], Japan[46]	R[6,20,43,46], *R/S[23]	8

150	80-bp deletion	Korea[20]	R	1
151	C>G	China[51]	R	1
	C>A	Brazil[17]	R	1
	C>T	Scotland[10]	R	1
	80-bp deletion	Korea[6]	R	1
152	A>C	Portugal[9], Korea[20], Thailand[22], Turkey[27], USA[33], Japan[46], China[47]	R[9,20,22,27,33,46,47]	10
	A>G	Brazil[11], Thailand[22], Peru[32], Italy[40], India[50], Canada[64]	R[11,22,32,40,50,64]	21
153	C>A	Japan[19], Sweden[38], USA[39], Poland[66]	R[19,38,39,66]	6
	C>G	Poland[19,66], India[58], Sweden[61]	R[19,58,61,66]	7
153 & 493	C>A & G>A	Japan[14]	R	1
156 & 546	C>A & G>T	India[50]	R	1
157	G>A	Japan[19]	R	1
158	A>C	Korea[12]	R	1
	44-bp deletion	China[47]	R	1
	A deletion	Korea[6]	R	1
160	C>A	China[15]	R	1
	C>T	Sweden[38,61]	R[38,61]	2
161	C>G	Japan[16], Canada[64]	R[16,64]	2
	C>T	Japan[14,19], Taiwan[18], South Africa[21,24], China[34,47], Peru[32], Sweden[38,61], Spain[59]	R[14,18,19,21,24,32,34,38,47,59,61]	15
	C>A	Brazil[11], Korea[20], China[34]	R[11,20,34]	3
	C deletion	Korea[6]	R	1
161 & 225	C>T & T>C	Mexico[26]	R	1
165	T>A	Taiwan[18]	R	1
166	G>T	Taiwan[18]	R	1
167	A>G	Taiwan[18]	R	1
168	C insertion	Taiwan[18]	R	1
169	C>G	Iran[2], Korea[6], Sweden[38,57], USA[39,55], New Zealand[45], Japan[46],	R[2,6,38,39,45,46,55,57,64]	21

		Canada[64]		
	C>T	South Africa[24], China[31,48], Netherlands[41]	R[24,31,41,48]	7
169 & 222	C>G & C>T	Canada[64]	R	1
170	A>C	Taiwan[18], China[34], Turkey[44], Japan[46]	R[18,34,44,46]	4
	A>G	Korea[5], Brazil[11], Canada[64]	R[5,11,64]	3
	T insertion	China[34]	R	1
171	C>T	Taiwan[18]	R	1
172	T>C	Korea[6,7], Peru[13], Sweden[38,61]	R[6,7,13,38,61]	6
	T deletion	South Africa[24]	R	12
	2-bp deletion	Brazil[23]	R	1
173	T>C	China[31,47]	R[31,47]	2
174	C>A	Taiwan[18], South Africa[30]	R[18,30]	2
	C>G	Korea[20], Canada[22,64], Sweden[38]	R[20,22,38,64]	4
	T insertion	China[34]	R	1
175	T>C	South Africa[3], Brazil[11,49], Japan[14], Korea[20], Turkey[44]	R[3,11,14,20,44,49]	6
180 & 425	C>T & C>T	Korea[7]	R	1
181	A>C	France[37]	R	1
181 & 416	A>C & T>C	Japan[46]	R	1
182	5-bp insertion	Brazil[17]	R	3
	CA deletion	Japan[46]	R	1
184	C>A	Latvia[25], New Zealand[45], China[47]	R[25,45,47]	3
184 & 290	T insertion & G>T	Japan[46]	R	1
185	C>T	Japan[16], Korea[20], Peru[32], China[63]	R[16,20,32,63]	5
	C>A	Azerbaijan[10], Taiwan[18], Poland[66]	R[10,18,66]	3
	C>G	USA[33]	R	1
188	A>G	Yemen[22], Latvia[25], Belgium[43], Canada[64]	R[22,25,43,64]	5
	A>C	Mexico[26], Belgium[43], Singapore[62]	R[26,43,62]	3
	2-bp insertion	Japan[16], Peru[32]	R[16,32]	2

	T insertion	New Zealand[45]	R	1
190	T>G	Korea[6,7]	R[6,7]	2
192	8-bp insertion	Brazil[23]	R	3
	A insertion	France[37], China[48]	R[37,48]	2
193	A insertion	Portugal[9], Canada[12], USA[12]	R[9, 12]	3
194 & 244	C>G & C>T	Turkey[44]	R	1
195	C>T	Iran[2], India[50], USA[39], Sweden[61]	R[2,39], *R/S[50,61]	19
	68-bp deletion	Azerbaijan[10]	R	1
195 & -11	C>T & A>G	India[50]	R	1
195 & 11	C>T & T>G	Canada[64]	R	1
195 & 29	C>T & A>G	India[50]	R	1
195 & 43	C>T & T deletion	Iran[2]	R	1
195 & 131	C>T & T>G	Iran[2]	R	1
195 & 182 & 226	C>T & A insertion & A deletion	Korea[1]	R	1
195 & 202	C>T & T>C	USA[39]	R	1
195 & 282	C>T & 2-bp deletion	Canada[64]	R	1
195 & 314	C>T & G>A	India[50]	R	1
195 & 328	C>T & G>T	India[50]	R	1
195 & 329	C>T & A deletion	Thailand[65]	R	1
195 & 391	C>T & G insertion	Canada[64]	R	1
195 & 406	C>T & G>A	India[50]	R	1
195 & 408	C>T & G insertion	Canada[64]	R	1
195 & 422	C>T & A>C	Canada[64]	R	1
195 &	C>T & T>C	India[50]	R	1

515				
195 & 528	C>T & GC insertion	Sweden[38]	R	1
195 & 535	C>T & A>C	Iran[2]	R	1
195 & 535	C>T & A deletion	India[50]	R	1
196	T>C	Korea[1], Russia[4], China[31]	R[1,4,31]	3
	14-bp insertion	Korea[20]	R	1
199	T>C	Korea[6], Thailand[8], Canada[22], Sweden[38], China[47,48], Canada[64]	R[6,8,22,38,47,48,64]	7
200	8-bp insertion	India[50]	R	1
200 & 390	C>T & GG insertion	Thailand[65]	R	1
202	T>C	Korea[1,6], Brazil[17], South Africa[24], Sweden[38], China[47,48]	R[1,6,17,24,38,47,48]	11
	T>G	Korea[6], South Africa[24], Mexico[26], Poland[66]	R[6,24,26,66]	10
203	G>T	Brazil[11], Hong Kong[28], Spain[59]	R[11,28,59]	5
	G>C	Korea[1,6]	R[1,6]	2
	G>A	Korea[6,20], USA[12]	R[6,12,20]	4
205	C>G	Brazil[23]	R	1
206	C>G	Korea[20], India[58]	R[20,58]	4
	C>T	France[37]	R	1
207	ACC deletion	Taiwan[18]	R	1
211	C>T	South Africa[3,24], Taiwan[18], Mexico[26], Peru[32], Belgium[43], Canada[64]	R[3,18,24,26,32,43,64]	8
	C>G	Thailand[8]	R	8
	C>A	China[34], Belgium[43]	R[34,43]	2
	3-bp deletion	Korea[20]	R	1
212	A>G	Iran[2], Korea[7], Portugal[9], USA[33]	R[2,7,9,33]	6
	A>C	Japan[46]	R	1
	14-bp insertion	Korea[20]	R	1
213	T>A	Korea[1], Netherlands[41]	R[1,41]	3
	T>G	USA[12]	R	1
	T deletion	Brazil[17]	R	1

214	T>C	China[15], Thailand[22], Brazil[23], India[58]	R[15,22,23,58]	6
215	G>A	Thailand[8], Brazil[23], South Africa[24]	R[8,23,24]	3
	29-bp deletion	France[37]	R	1
216	C>A	Korea[20], China[47]	R[20,47]	2
	C>G	Japan[19], Latvia[25], Canada[64]	R[19,25,64]	4
218	G insertion	Netherlands[41]	R	1
221	G insertion	Scotland[10]	R	2
222	G>C	Thailand[8]	R	1
223	T>C	South Africa[21]	R	1
225 & 515	T>C & T>C	Mexico[26]	R	1
226	A>C	Korea[1,6,20], Brazil[17,23], South Africa[21], Myanmar[22], Latvia[25], Mexico[26], China[31,34,47,63], Peru[32], USA[33], Portugal[35], Netherlands[41], India[50], Cuba[56], Spain[59], Canada[64]	R[1,6,17,20,21,22,23,25,26,31,32,33,34,35,41,47,50,56,59,63,64]	38
	A>G	Taiwan[18]	R	1
227	C>T	Korea[6,7], Brazil[23], Turkey[44]	R[6,7,23,44]	4
232	G>A	Korea[5]	R	1
	G>T	Peru[32]	R	1
233	G>T	Taiwan[18]	R	1
	G>A	Korea[6], USA[33]	R[6,33]	2
	G insertion	USA[39]	R	1
233-237	GCGCG-ACGTCTCCA substitution	Korea[20]	R	1
234	G insertion	China[34]	R	1
	4-bp insertion	Korea[5]	R	1
236 & 360	C>T & T insertion	South Africa[3]	R	1
239	A deletion	Turkey[44]	R	1
241	T>G	China[31,63]	R[31,63]	3
244	C>G	Belgium[43]	R	1
245	A>G	USA[12], Brazil[17], China[34,63],	R[12,17,43,45,63], *R/S[34]	7

		Belgium[43], New Zealand[45]		
	AT>GG	China[31]	R	1
245 & 389	A>G & T>C	Korea[20]	R	1
246	A>T	Brazil[17]	R	1
248	C>G	Taiwan[18]	R	1
249	T>G	China[34]	R	1
250	C insertion	Portugal[9,35]	R[9,35]	7
254	T>C	Iran[2], Korea[6], China[15], Taiwan[18], Peru[32], USA[33,39], India[58]	R[2,6,15,18,32,33,39,58]	15
	T>G	Korea[6,12], Azerbaijan[10], China[15], Japan[16], Latvia[25]	R[6,10,12,15,16,25]	7
	T>A	Canada[64]	R	2
258	18-bp insertion	Peru[32]	R	1
260	C>T	Belgium[43]	R	2
	C deletion	Canada[64]	R	1
	AC insertion	Japan[46]	R	1
260 & 436	C>T & G deletion	France[37]	R	1
261	2-bp insertion	Japan[14,16]	R[14,16]	4
263	C>A	China[15], Canada[64]	R[15,64]	2
264	24-bp deletion	USA[55]	R	1
269	T>G	South Africa[24], Sweden[38], USA[55], Canada[64], Thailand[65]	R[24,38,55,64,65]	5
279	106-bp insertion	Peru[32]	R	1
280	T>C	China[31], Peru[32]	R[31,32]	4
281	T>C	Mexico[26], China[31]	R[26,31]	2
	T>G	Cuba[56]	R	1
282	C>G	Korea[1], Thailand[65]	R[1,65]	2
	T insertion	Portugal[35]	R	1
285	C>A	Portugal[35]	R	1
	CA deletion	Peru[32]	R	2
286	A>G	Russia[4], Korea[6,20], Portugal[9], China[31], India[50]	R[4,6,9,20,31,50]	14
	A>C	Korea[6]	R	1
	A>T	South Africa[3]	R	1

	A deletion	Korea[20]	R	2
287	A>C	Brazil[11,49], Korea[20], China[34], France[37]	R[11,20,34,3749]	7
	A>G	Mexico[26]	R	1
	A>T	Italy[40]	R	1
	T insertion	Korea[6], Canada[22]	R[6,22]	2
288	G>T	China[15]	R	2
	G>C	India[58]	R	37
	G deletion	USA[39]	R	1
289	G>T	South Africa[3,21], Italy[40], India[50]	R[3,21,40,50]	6
	G>A	Thailand[8], USA[12,33], Korea[20], China[34]	R[8,12,20,33,34]	8
	G>C	Netherlands[41]	R	1
290	G>A	South Africa[3], Japan[16], Taiwan[18,29], USA[33], China[34], New Zealand[45], Singapore[62]	R[3,16,18,29,33,34,45,62]	8
	T insertion	Portugal[9]	R	1
291	T>C	Sweden[57]	R	2
	T deletion	Italy[40]	R	1
292	G insertion	Poland[66]	R	1
293	10-bp deletion	Peru[32]	R	1
297	C>G	Brazil[17], USA[33]	R[17,33]	2
	C>A	Thailand[22], Japan[46]	R[22,46]	2
298	A>C	China[34]	R	4
	A>G	Taiwan[18]	R	1
300 & 539	C deletion & T>C	Singapore[62]	R	1
301	G deletion	Korea[12]	R	1
	A insertion	China[15]	R	1
303	A deletion	Korea[20]	R	1
304	G>A	Russia[4]	R	1
305	C>T	South Africa[3,60], Russia[4], China[34]	R[3,4,34,60]	8
306	C insertion	France[37]	R	1
307	T>G	Canada[64]	R	1
	T>C	Korea[6], Canada[10], Latvia[25], Sweden[38]	R[6,10,25,38]	10
	5-bp deletion	Korea[6]	R	1

308	A>T	Latvia[25]	R	1
	A>G	Taiwan[18], China[34], France[37], Poland[66]	R[18,34,37,66]	4
	A>C	Korea[6]	R	2
309	C>G	Russia[4], Korea[6], Brazil[17], South Africa[24], Peru[32], USA[33]	R[4,6,17,24,32,33]	15
	C>A	Korea[1], USA[33], Cuba[56]	R[1,33,56]	4
310	A>T	USA[55]	R	1
312	C>A	Korea[1,20]	R[1,20]	2
	C>G	Thailand[8,65], Yemen[22]	R[8,22,65]	4
314	G>A	Brazil[11], Peru[32], China[34]	R[11,32,34]	4
	C insertion	Brazil[11]	R	2
315	G insertion	South Africa[3], Brazil[17], China[34]	R[3,17,34]	4
316	245-bp deletion	Poland[66]	R	1
317	CT insertion	Korea[7]	R	1
319	G>A	South Africa[24]	R	1
	G>T	Taiwan[18]	R	1
322	G>T	Iran[2]	R	1
	G>C	Iran[2], Brazil[11], Korea[20]	R[2,11,20]	4
334	A>T	China[34]	R	1
335	A>T	Taiwan[18]	R	2
337	G deletion	Korea[20]	R	1
338 & 472	G>A & G>A	Korea[5]	R	1
339	G insertion	Canada[64]	R	1
340	A>C	Yemen[22], South Africa[24], Poland[66]	R[22,24,66]	4
341	C deletion	Korea[6,12]	R[6,12]	2
	1355-bp deletion	France[37]	R	1
346	C>G	South Africa[24]	R	1
347	T>G	Korea[1], Russia[4], India[50], Poland[66]	R[1,4,50,66]	11
	T>C	Peru[32], Italy[40], China[63]	R[32,40], *R/S[63]	4
350	T insertion	Japan[46]	R	1
352	7-bp deletion	Taiwan[29]	R	1
353	A>C	China[15]	R	1
355	T>A	China[34]	R	2
356	G>T	Peru[32]	R	1

357	G>A	Korea[6], Cuba[56]	R[6,56]	2
359	T>G	Iran[2], Brazil[11,49], New Zealand[45], India[50], Spain[59]	R[2,11,45,49,50,59]	6
	T>C	Portugal[9,35], Mexico[26], Sweden[57]	R[9,26,35,57]	29
	81-bp deletion	Peru[32]	R	2
359 & 435	T>G & G insertion	Korea[1]	R	1
361	C deletion	Spain[59]	R	1
362	G>C	Korea[6], China[15,48]	R[6,15,48]	3
	G>A	Thailand[8]	R	1
364	C>T	Taiwan[18], South Africa[21,60], Peru[32]	R[18,21,32,60]	4
368	G>C	Sweden[38,57,61]	R[38,57,61]	3
	G>T	South Africa[21]	R	1
	18-bp insertion	Korea[6]	R	1
	AG insertion	China[15]	R	1
373	G>T	Thailand[8], Sweden[38,61], New Zealand[45]	R[8,38,45,61]	4
	9-bp insertion	Korea[20]	R	1
374	T>G	Portugal[9,35], Mexico[26]	R[9,26,35]	28
	T>A	Russia[4], Sweden[38]	R[4,38]	3
	16-bp deletion	Japan[14]	R	1
375	C insertion	Mexico[26]	R	1
	9-bp deletion	Spain[59]	R	1
377	GA insertion	Peru[32]	R	1
378	10-bp deletion	China[47]	R	1
379	G>A	Iran[2]	R	1
	G>T	Thailand[8], China[34]	R[8,34]	2
	30-bp deletion	Mexico[26]	R	1
	11-bp deletion	Russia[4]	R	1
380	9-bp deletion	Mexico[26]	R	1
	11-bp deletion	Korea[20]	R	1
381	GG deletion	Korea[6]	R	1
	9-bp deletion	Portugal[35]	R	1
382	AG insertion	Korea[6,7]	R[6,7]	2
	8-bp deletion	Japan[36]	R	1
	9-bp deletion	India[50], Cuba[56]	R[50,56]	2

383	T>G	New Zealand[45], Japan[46], Sweden[57]	R[45,46,57]	3
	9-bp deletion	USA[39]	R	1
385	5-bp deletion	Korea[20]	R	1
	CG deletion	Japan[46]	R	1
385 & 420	T>G & GG insertion	USA[12]	R	1
386	T>G	Brazil[17]	R	1
	4-bp deletion	Korea[6]	R	1
388	9-bp insertion	Brazil[23], USA[33]	R[23,33]	2
389	T>C	Korea[1], China[34,47]	R[1,34,47]	4
	T>G	Canada[22], USA[33], Thailand[65]	R[22,33,65]	4
	G insertion	India[50]	R	1
	GG insertion	Korea[20], South Africa[24], India[50]	R[20,24,50]	12
390	GG insertion	Mexico[26]	R	1
391	G>T	Korea[20]	R	1
	GG insertion	Korea[6], Portugal[9], France[37], Sweden[38,57], Japan[46], China[47]	R[6,9,37,38,46,47,57]	8
	G insertion	Mexico[26], China[47], Poland[66]	R[26,47,66]	3
392	G insertion	Korea[6], Portugal[35]	R[6,35]	2
	GG insertion	Canada[64]	R	1
393	GT insertion	Korea[7]	R	1
	T insertion	Korea[7]	R	1
	1-bp deletion	Korea[1]	R	1
394	G>A	South Africa[3], Taiwan[18], Japan[19], China[34], Sweden[38,61]	R[3,18,19,34,38,61]	7
395	G>T	Iran[2], Korea[7]	R[2,8]	3
	G>A	Portugal[9], Taiwan[18], Korea[20], Myanmar[22], France[37], Canada[64]	R[9,18,20,22,37,64]	7
	G>C	China[31], Sweden[38,57,61], India[50]	R[31,38,50,57,61]	5
	9-bp insertion	Canada[64]	R	1
	17-bp deletion	India[22]	R	1
396	T insertion	China[48]	R	1
	TC insertion	India[58]	R	3
397	G insertion	Taiwan[29]	R	1
398	T>C	Russia[4], Japan[19], Portugal[35], China[63]	R[4,19,35,63]	5

	T>G	Brazil[11]	R	1
	T>A	USA[33]	R	1
	TT deletion	South Africa[21]	R	1
399	163-bp deletion	Singapore[62]	R	1
401	C>T	Canada[12], Peru[13], Taiwan[18,29], USA[33]	R[12,13,18,29,33]	5
403	A>C	Korea[1,6,7,20], China[31], Peru[32], USA[33], Japan[46], Poland[66]	R[1,6,7,20,31,32,33,46,66]	22
	A>G	Taiwan[18]	R	1
	CC insertion	China[15]	R	1
404	C>A	Poland[66]	R	3
406	G>A	Korea[6], Brazil[54]	R[6,54]	3
	G>C	Portugal[9], Scotland[10]	R[9,10]	2
	G>T	Korea[6]	R	1
	G deletion	Korea[6]	R	1
407	A>G	Korea[7,20], South Africa[24], China[31,63]	R[7,20,24,31], *R/S[63]	6
	C insertion	Azerbaijan[10]	R	1
	GC insertion	Japan[46]	R	1
408	A insertion	Korea[1], China[63], Canada[64]	R[1,63,64]	3
410	A>C	Korea[6], Bangladesh[10], Japan[14], Peru[32], China[47], India[58]	R[6,10,14,32,47,58]	7
	A>G	USA[33]	R	2
412	T>C	Japan[16,46]	R[16,46]	2
	G insertion	Thailand[8]	R	1
	T deletion	Japan[46]	R	1
413	G>A	China[15], Latvia[25], USA[33], Sweden[38], India[58]	R[15,25,33,38,58]	6
	G>C	Korea[12]	R	1
414	T>A	Korea[20]	R	2
	T>C	Brazil[17]	R	2
	G insertion	Korea[6]	R	1
415	G>C	China[15,31], Mexico[26], USA[33], India[58]	R[15,26,31,33,58]	6
	G>A	Korea[6,12], South Africa[24]	R[6,12,24]	6
	G>T	Japan[46]	R	1
415 &	G deletion & C>T	India[50]	R	1

418				
416	T>C	China[15,31,47], Taiwan[18], Korea[20], Malaysia[22], South Africa[30], Japan[53], Cuba[56], Poland[66]	R[15,18,20,22,30,31,47,53,56,66]	13
	T>G	Thailand[8], China[31,34,48], USA[33], Portugal[35], Spain[59], South Africa[60], Singapore[62]	R[8,31,33,34,35,48,59,60,62]	13
418	G insertion	Korea[1,20]	R[1,20]	3
418 & 446	C>A & 8-bp deletion	Canada[12,64]	R	24
419	68-bp deletion	France[37]	R	1
420	G insertion	USA[12]	R	2
	GC insertion	South Africa[21]	R	1
421	C>T	Korea[6,7], Portugal[9]	R[6,7,9]	3
422	A>C	Korea[1,6,20], Russia[4], USA[12], China[31,47], India[58]	R[1,4,6,12,20,31,47,58]	10
	C deletion	Brazil[17]	R	1
424	A>G	Myanmar[22], China[34,47], Poland[66]	R[22,34,47,66]	4
425	C>A	China[15], Thailand[22], Sweden[38,61]	R[15,22,38,61]	7
	C>T	Thailand[8], Bangladesh[10],Korea[20], New Zealand[45], India[58]	R[8,10,20,45,58]	6
426	AG insertion	India[58]	R	1
427	G>A	Iran[2]	R	1
	G>C	Mexico[26]	R	1
428	C>G	Italy[40]	R	1
	GG insertion	Korea[12]	R	1
430	GAG deletion	Netherlands[41]	R	1
	6-bp deletion	Poland[66]	R	2
436	G>A	Korea[6,20], Thailand[8,52], China[63]	R[6,8,20,52,63]	7
437	C>T	India[22]	R	1
439	CG insertion	Portugal[9,35]	R[9,35]	4
440	1-bp deletion	Mexico[26]	R	1
442	C>A	Poland[19], Turkey[44]	R[19,44]	2
	C>T	Taiwan[18]	R	1
444	CG insertion	Brazil[23]	R	1
	12-bp insertion	Sweden[38,57]	R[38,57]	2
448	GC insertion	Korea[20]	R	2

449	G deletion	Japan[46]	R	1
451	T deletion	Korea[20]	R	1
452	T>C	South Africa[3], China[34,63], Canada[64]	R[3,34,63,65]	6
	T deletion	Korea[6]	R	1
454	G>A	Korea[5]	R	1
456	T insertion	Brazil[54]	R	2
457	C>G	Brazil[23]	R	1
458	C>A	USA[33]	R	1
	C>T	China[47]	R	2
458 & 474	C>T & C>A	China[48]	R	1
460	A>G	Korea[1], South Africa[3,60], Russia[4], China[34]	R[1,3,4,34,60]	5
461	G>C	Taiwan[18]	R	1
463	G>T	Korea[20]	R	1
	G>A	New Zealand[45], Canada[64]	R[45,64]	3
464	T>G	Iran[2], Korea[6,12], Brazil[11,17,49], Taiwan[29], France[37], Sweden[38,57,61], Belgium[43], Cuba[56]	R[2,6,11,12,17,29,37,38,43,49,56,57,61]	18
	T>C	Korea[20]	*R/S	1
	T insertion	Italy[40]	R	2
465	T insertion	Korea[6]	R	2
467	T>A	Japan[46]	R	1
470	T>G	Korea[1]	R	1
	T>C	Poland[66]	R	2
472	T>G	Thailand[65]	R	1
476	T>C	South Africa[3,24], Portugal[9], China[34]	R[3,9,24,34]	11
	T>G	Korea[6,20], China[31]	R[6,20,31]	18
478	A>C	Iran[2], Russia[4]	R[2,4]	3
479	C>A	Mexico[26], Turkey[27]	R[26,27]	2
480	TGAC insertion	Korea[6]	R	1
481	G>C	Russia[4]	R	2
484	G>A	China[63]	R	1
484 & 494	9-bp deletion & 2-bp deletion	Japan[16]	R	1

485	G>A	Brazil[17], Hong Kong[28], China[31], USA[39]	R[17,28,31,39]	5
	GT deletion	Korea[20]	R	1
486	T insertion	Poland[66]	R	1
	10-bp deletion	Japan[46]	R	1
487	10-bp deletion	Mexico[26]	R	1
488	T>C	India[50]	R	1
489	T insertion	Korea[1]	R	1
490	T>C	Brazil[11,49]	R[11,49]	2
	T deletion	Peru[32]	R	5
495	C insertion	Brazil[11]	R	1
497	1-bp insertion	Japan[16]	R	1
499	A>C	China[47]	R	1
501	CG insertion	China[15]	R	1
502	A>C	Korea[20], China[31], Netherlands[41], Spain[59]	R[20,31,41,59]	6
503	C>A	USA[12]	R	1
511	G>A	Portugal[9], Bangladesh[10], USA[33]	R[9,10,33]	3
	G>C	India[58]	R	1
512	C>A	Korea[1], Brazil[17], China[48]	R[1,17,48]	5
	C>T	China[63], Canada[64]	*R/S[63], R[64]	3
	C deletion	Rwanda[10]	R	1
513	GC deletion	Korea[7]	R	1
514 & 515	C>G & T>C	Belgium[10]	R	2
515	T>C	Korea[6], Canada[22], Brazil[23], USA[33,39], Sweden[38,61], China[48], Cuba[56], South Africa[60]	R[6,22,23,33,338,39,48,56,60,61]	15
	T>G	Belgium[43], Poland[66]	R[43,66]	5
	T deletion	South Africa[3]	R	1
516	G insertion	Netherlands[41]	R	1
520	G>T	Korea[20]	R	2
521	A>G	China[31]	R	1
	GG insertion	Thailand[8]	R	1
	T insertion	Peru[32]	R	1
523	A>G	Japan[19], Brazil[23], Belgium[43], Poland[66]	R[19,23,43,66]	7

524	T>G	Korea[20]	R	1
	T>C	Sweden[38,61]	R[38,61]	2
525	G>A	China[31], Belgium[43], Brazil[54]	R[31,54], *R/S[43]	4
529	A>C	Taiwan[18]	R	1
530	C deletion	Belgium[43]	R	2
531	6-bp deletion	Poland[66]	R	3
532	C insertion	Korea[6]	R	1
535	A>C	Iran[2], South Africa[24]	R[2,24]	3
537	G>T	Latvia[25]	R	1
538	G>T	Korea[6], China[47], India[50], Canada[64]	R[6,47,50,64]	5
542	A deletion	China[34]	R	1
543	G>T	South Africa[24]	R	1
545	T>C	France[37]	R	1
551	G>A	Taiwan[18]	R	1
554	G>C	South Africa[24]	R	8
559	T>G	India[50]	R	1
564	G>C	Brazil[54]	R	1
572	G>A	Iran[2]	R	1
573	A>C	Canada[64]	R	1
582	T deletion	South Africa[3]	R	1

Abbreviations: A, adenine; bp, base pair; C, cytosine; del, deletion; G, guanine; R, resistant; SNP, single-nucleotide polymorphism; T, thymine.

* Article found one isolate sensitive and one isolate resistant.

Supplementary references

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Chapter 3

Prevalence of pyrazinamide resistance across the spectrum of drug resistant phenotypes of *Mycobacterium tuberculosis*

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Abstract

Pyrazinamide resistance is largely unknown in the spectrum of drug resistant phenotypes. We summarize data on PZA resistance in clinical isolates from South Africa. PZA DST should be performed when considering its inclusion in treatment of patients with rifampicin-resistant TB or MDR-TB.

Text

Pyrazinamide (PZA) is a critical component of the first-line tuberculosis (TB) treatment regimen [1]. The unique ability of PZA to target subpopulations of persister bacteria has significantly reduced the treatment duration from 9-12 to 6 months [2]. The importance of PZA is further highlighted by its inclusion in the latest WHO recommended multi-drug resistant (MDR)-TB treatment regimen [3] as well as most novel MDR-TB regimens presently under investigation [4].

Little is known regarding the prevalence of PZA drug resistance, largely because drug susceptibility testing (DST) of PZA is not yet standardized and thus not routinely implemented in high burden countries. The BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system PZA (BD Diagnostic Systems, NJ, USA) recently became the reference technique, but concerns remain since false resistance may occur with this system [5]. False resistance may occur due to inoculum size or the presence of bovine serum albumin in the media of the MGIT 960 DST.[6]

Mutations in *pncA* are the primary mechanism of resistance to PZA [7]. It is hypothesized that these mutations decrease pyrazinamidase (PZase) activity, thereby limiting the conversion of PZA to the active form of pyrazinoic acid (POA) [7]. A high sensitivity (92%) and specificity (93%) for PCR-DNA sequencing when compared to the phenotypic DST supports the use of genotypic DST for the identification of PZA resistance [8]. Extensive DNA sequencing studies of *pncA* have revealed that mutations occur across the entire length of the gene, making it a complex target for a molecular diagnostic to identify all causal mutations [9, 10]. Furthermore, not all non-synonymous mutations cause phenotypic resistance [6], and mutations in the *pncA* gene are absent in a small percentage of phenotypically PZA resistant isolates [11] suggesting that resistance could be conferred via an alternative mechanism(s), such as the gene *rpsA* or *panD* [12, 13].

Several studies have investigated the prevalence of PZA resistance in drug susceptible as well as MDR-TB isolates. In a recent meta-analysis, the prevalence of PZA resistance was 0 – 9% among drug susceptible and 31 – 89% among MDR-TB isolates [8]. Data is scarce on the prevalence of PZA resistance in RIF mono-resistant, INH mono-resistant, extensively drug resistant (XDR) and pre-XDR TB isolates.

This study aimed to determine the association between mutations in the *pncA* gene and phenotypic resistance to PZA and to investigate the prevalence of PZA resistance across the spectrum of drug resistant isolates, from pan-susceptible TB to XDR-TB.

Materials and Methods

Study samples

A convenience sample of 775 drug resistant clinical isolates classified as INH mono-resistant, RIF mono-resistant or MDR-TB by the National Health Laboratory Services (NHLS) was selected. Isolates came from across South Africa (Western Cape, Eastern Cape, Free State and Gauteng) and duplicates were excluded. Routine DST for INH and RIF at the NHLS was done using either MGIT culture or the GenoType MTBR*plus* (Hain LifeScience) line probe assay (LPA).

Phenotypic DST of INH, RIF and PZA

At the MRC Centre for Tuberculosis Research laboratory, all selected isolates were subjected to phenotypic INH, RIF and PZA susceptibility testing using the BACTEC MGIT 960 method (BD Diagnostic Systems, NJ, USA). Growth at a critical concentration of 0.1µg/ml INH, 1.0µg/ml RIF, and 100µg/ml PZA was used to define resistance [14].

Genotypic classification of isolates

DNA sequencing of the *gyrA* and *rrs* gene was done to identify pre-XDR-TB and XDR-TB, as previously described [15]. DNA sequencing of the *pncA*, *panD* and *rpsA* genes were done to identify PZA resistance, as previously described [12, 13, 16]. No restriction fragment length polymorphism (RFLP) genotyping was performed.

Statistics

We used the Cochran-Armitage test in R to assess the presence of a trend in prevalence of PZA resistance by levels of resistance to other drugs.

Results

Phenotypic and genotypic characterisation of isolates

Based on phenotypic and genotypic DST performed at the research laboratory, 80 (10.3%) isolates were classified as pan-susceptible, 98 (12.7%) as INH mono-resistant; 279 (36.0%) as RIF mono-resistant, 224 (28.9%) as MDR-TB and 94 (12.1%) as pre-XDR and XDR-TB. Of the 80 pan-susceptible and 98 INH mono-resistant samples, 96 were confirmed rifampicin susceptible by molecular methods (MTBDRplus, Xpert MTB/RIF, *rpoB* sequencing). Low level rifampicin resistance may have been missed among the 82 samples evaluated only by MGIT960 DST, possibly resulting in some misclassification of samples as rifampicin susceptible.

Correlation of genotypic and phenotypic PZA resistance by level of resistance to other TB drugs

Overall, 246 (31.7%) isolates had a polymorphism in the *pncA* gene: 29 (11.8%) synonymous single nucleotide polymorphisms (SNP), 126 (51.2%) non-synonymous SNPs, 80 (32.5%) insertions and 11 (4.5%) deletions. Among all 775 isolates, 15 had discordant results between genotypic and phenotypic DST after considering SNPs known to not confer resistance to PZA at 100µg/ml [6]. Most discrepancies (10/15) were phenotypically resistant despite the presence of wild type *pncA* gene. The remaining 5 discrepancies were phenotypically susceptible with a non-synonymous change in the *pncA* gene (TTC13TGC, TGC72GGC, GCG102GTG, GTG139GGG). Using the phenotypic DST results as the reference standard, genotypic DST had an overall sensitivity of 95.0 (95% CI 92.1 - 98.0) and specificity of 99.1 (95% CI 98.4 – 99.9). The strong correlation between phenotypic PZA-resistance and changes in the *pncA* gene was observed independent of the resistance profile to other TB drugs. DNA sequencing of the *panD* gene failed to identify any mutations, sequencing of the *rpsA* gene identified one synonymous mutation (CGA212CGC) not associated with resistance [12].

Phenotypic PZA resistance by level of resistance to other TB drugs

Using the BACTEC MGIT 960 system, the number of isolates resistant to PZA at critical concentration of 100µg/ml was: 0 of 80 (0.0%) pan-susceptible isolates, 2 of 98 (2.0%; 95% CI 0, 4.8) INH mono-resistant isolates, 21 of 279 (7.5%; 95% CI 4.4, 10.6) RIF mono-resistant isolates, 88 of 224 (39.3%; 95% CI 32.9, 45.7) MDR isolates, and 91 of 94 (96.8%; 95% CI 93.2, 100) pre-XDR-TB and XDR-TB isolates (Figure 1). The proportion of isolates with PZA resistance increased significantly with increasing resistance to other drugs (Cochran-Armitage test, p =< 0.0005).

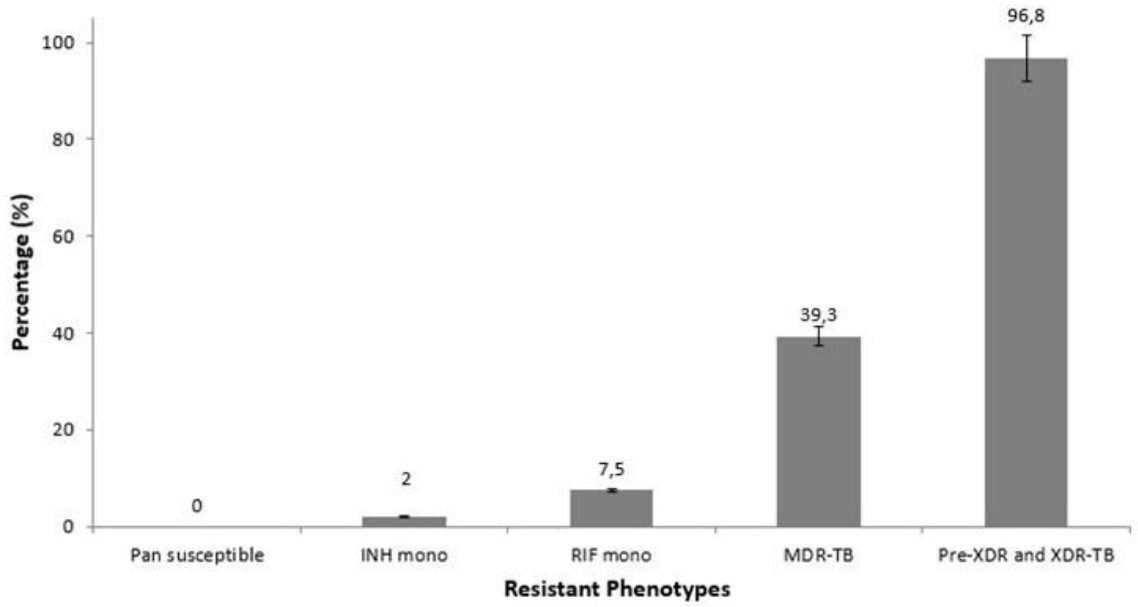

Figure 1. PZA resistance prevalence in different resistant phenotypes.

Discussion

We found a significant increase in the prevalence of PZA resistance with increasing level of resistance (pan-susceptible (0%) to pre-XDR and XDR-TB (96.8%)). These findings extend prior observations of low prevalence of PZA resistance in pan-susceptible but high prevalence of PZA resistance in MDR-TB isolates [8, 10]. Our findings strongly suggest that patients with pan-susceptible and INH mono-resistance benefit from inclusion of PZA in their treatment regimen and that PZA DST should be routinely performed in patients with RIF mono-resistance and MDR-TB. Lastly that the inclusion of PZA in treatment regimens for pre-XDR and XDR regimen is likely to cause more harm due to

side effects than benefit since 97% (95% CI 93, 100) of all pre-XDR and XDR-TB isolates were resistant to PZA.

Similar to other studies, we found an excellent correlation between phenotypic and genotypic DST with an overall sensitivity of 95.0% (95% CI 92.1 - 98.0) and a specificity of 99.1% (95% CI 98.4 – 99.9). Only DST results generated in our laboratory were considered, I did not consider publications which reported resistance for certain mutations which I found to be susceptible. We further observed that this strong correlation was independent of the resistance profile to other TB drugs. Taken together, these observations provide mounting evidence supporting the use of genotypic instead of the slow and cumbersome phenotypic DST for PZA [10]. Use of genotypic DST could however result in some misclassification due to the existence of silent mutations and resistance not conferred by mutations in the *pncA* gene [6]. In our study, the occurrence of discordances was rare, similar to other studies findings [11]. Finally, the role of some *pncA* mutations is unclear as two mutations (GCG102GTG, GTG139GGG) were found to be susceptible in our study but resistant in another study [10].

Conclusion

The prevalence of PZA resistance is strongly correlated with resistance to rifampicin and increases with additional resistance in MDR and XDR-TB strains. The presence of rifampicin-resistance could then be considered as an indicator to perform DST to PZA. Consideration should also be given when defining the role of PZA in future TB treatment regimens.

Conflict of Interest: None

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Chapter 4

Mycobacterium tuberculosis pncA Polymorphisms That Do Not Confer Pyrazinamide Resistance at a Breakpoint Concentration of 100 Micrograms per Milliliter in MGIT

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- Interpretation of results and data
- Writing and editing of manuscript
- Addressed reviewer's comments

Abstract

Sequencing of the *Mycobacterium tuberculosis pncA* gene allows for pyrazinamide susceptibility testing. We summarize data on *pncA* polymorphisms which do not confer resistance at a susceptibility breakpoint of 100 µg/ml pyrazinamide in MGIT within a cohort of isolates from South Africa and the US Centre for Disease Control.

Text

Culture-based drug susceptibility testing (DST) using the BACTEC MGIT PZA 960 medium (Becton Dickinson, Sparks, MD) at 100 µg/ml is the current gold standard test for pyrazinamide (PZA) resistance (1). False resistance results are known to occur with this assay (1, 2), which could be the result of alkalinisation of the medium due to a high inoculum size or the presence of bovine serum albumin (3). The use of alternative susceptibility breakpoint concentrations or different media are additional factors that may contribute to disparities in PZA susceptibility results (4-6). A further limitation of culture-based methods is the long turnaround time, which can exceed 20 days (7-9). Molecular methods offer an alternative strategy for the detection of PZA susceptibility. These methods detect polymorphisms in the 561bp *pncA* gene, which encodes the pyrazinamidase (PZase) enzyme that is responsible for conversion of PZA (pro-drug) to pyrazinoic acid (active form) (10). More than 325 polymorphisms (single nucleotide polymorphisms (SNPs), insertions and deletions) throughout the entire length of the *pncA* gene and in the promoter region have been described, complicating molecular detection (11-14). A good correlation (sensitivity >90%) between *pncA* polymorphisms in circulating isolates and phenotypic susceptibility results have been observed for PZA (15-18). Incomplete correlation of *pncA* molecular results with culture-based PZA testing has been ascribed to poor reproducibility of the phenotypic test or the presence of alternative genetic mechanisms for resistance including polymorphisms in the *rpsA* gene (19, 20). Additionally, a few *pncA* mutations have been reported in the absence of phenotypic resistance (15), but the role of such mutations in PZA resistance has not been thoroughly investigated. This study aimed to collate data on *pncA* polymorphisms in clinical isolates which do not confer resistance at a susceptibility breakpoint of 100 µg/ml PZA. To capture the spectrum of *pncA* mutations not associated with phenotypic PZA

resistance, we performed a comprehensive literature search. In the 77 papers reporting both genotypic and phenotypic PZA susceptibility (Supplemental Table S1), 77 different *pncA* polymorphisms in 71 different codons were reported to have a PZA susceptible phenotype using either BACTEC MGIT PZA 960 (Becton Dickinson, Sparks, MD), BACTEC PZA 460 (Becton Dickinson, Sparks, MD) or the Wayne's assay (21). Forty-seven (68.1%) of these polymorphisms have also been reported in PZA-resistant isolates. These inconsistent phenotypic results could due to technical difficulties of phenotypic PZA assays or MICs that are close to the breakpoint. Another 26 (33.7%) mutations were found in only one or two isolates suggesting these new mutations need to be characterized further to determine their role in PZA resistance.

To further investigate the relationship between *pncA* mutation and PZA susceptibility we analysed clinical isolates from culture collections at the Centers for Disease Control and Prevention (CDC), Atlanta, USA (n = 185) and Stellenbosch University, South Africa (n = 865). For the CDC isolates, *pncA* had previously been sequenced and only isolates with mutant *pncA* were included in this study. For the SA isolates, the *pncA* gene was amplified and sequenced using the ABI3130XL genetic analyzer (Applied Biosystems, Foster City, CA 94404 USA). Polymorphisms in *pncA* were identified in 231 (26.7%) of the SA clinical isolates relative to the PZA susceptible H37Rv reference strain. All isolates harbouring *pncA* mutations (CDC and SA) were subjected to DST against PZA (BD PZA Kit) at a critical concentration of 100 µg/ml using BACTEC MGIT 960. This phenotypic testing identified 7 of 185 (3.8%) CDC isolates and 42 of 231 (18.2%) SA isolates to have a susceptible PZA phenotype despite the presence of mutant *pncA* alleles. These results were confirmed by repeat *pncA* sequencing and repeat PZA DST. From these 49 isolates, 10 different *pncA* polymorphisms (synonymous, n = 2; non-synonymous, n = 8) were identified (Table 1). Six polymorphisms were newly identified in this study, while 4 corresponded to previously described *pncA* polymorphisms. Of these four, only Thr114Met was previously observed exclusively in susceptible isolates. The polymorphisms were not restricted to a defined domain but were broadly distributed throughout the *pncA* gene, with a distribution similar to that of resistance-causing mutations (22).

To further explore PZA resistance, each of the 49 PZA-susceptible isolates identified in this study was subjected to PZA minimum inhibitory concentration (MIC) determination using PZA concentrations

of 25, 50, 75 and 100 µg/ml in BACTEC MGIT PZA 960 medium. Six of the polymorphisms (all non-synonymous SNPs) showed an MIC between 50 and <100 µg/ml, 3 polymorphisms (2 were synonymous) were associated with an MIC < 25 µg/ml, and one polymorphism had an MIC<25 for one isolate and >50 for a second isolate (Table 1). It is important to note that most of the *pncA* polymorphisms associated with susceptible isolates (7/10) identified in this study had a PZA MIC between 50 and 100 µg/ml. Six of 10 *pncA* polymorphisms associated with susceptibility were present in more than one isolate. The reproducibility of the MIC determinations across different clinical isolates with the same *pncA* mutation supports the notion that these polymorphisms do not confer resistance above the breakpoint concentration. However, some of SNPs identified in this study were reported to confer resistance in other studies (Supplemental Table S1: References 1, 2, 24, 36, 50, 76). These conflicting results may be due to the PZA MICs for these isolates being close breakpoint or associated with technical difficulties of performing PZA DST on solid media (1, 2).

We acknowledge that the clinical relevance of these polymorphisms on treatment outcome remain to be determined. In a recent report, a MIC > 50 µg/ml and < 100 µg/ml was associated with a poor 2 month sputum conversion (relative risk of 1.5 (95% CI: 1.2-1.8)) compared with a MIC ≤ 50 µg/ml (23). Accordingly, the authors concluded that a PZA susceptibility breakpoint of ~50 µg/ml should be used for clinical decision making. However, one cannot exclude other factors which could have contributed to the observed delayed treatment response (23).

Based on the current accepted PZA susceptibility breakpoint concentration of 100 µg/ml (1), not all *pncA* mutations necessarily confer resistance. We propose that genetic PZA drug susceptibility testing results should be interpreted based on known phenotype and genotype relationships at a susceptibility breakpoint of 100 µg/ml until further evidence is presented in support of any revision of the susceptibility breakpoint. Further studies are required to improve our understanding of the relationship between treatment outcome and *pncA* mutations.

Table 1: Single nucleotide polymorphisms in *pncA* found to not be associated with PZA drug resistance within a cohort of South African and US isolates.

Codon (nucleotide position)	**Nucleotide change**	**Amino acid change**	**Associated MIC**	**Source of Isolate**	**Number of isolates**
35 (104bp)	CTG – CGG	Leu – Arg†	>25 < 75 µg/ml	Stellenbosch	13
37 (110bp)	GAA – GTA	Glu – Val	50 µg/ml	CDC	2
65 (195bp)	TCC – TCT	Ser – Ser†	<25 µg/ml	Stellenbosch and CDC	21
96 (288bp)	AAG – AAA	Lys – Lys	<25 µg/ml	Stellenbosch	2
110 (329bp)	GAC – GGC	Asp – Gly	50 µg/ml	CDC	1
114 (341bp)	ACG – ATG	Thr – Met†	<25 µg/ml	Stellenbosch	5
*130 (389bp)	GTG – GCG	Val – Ala†	<25 µg/ml and >50 <100 µg/ml	Stellenbosch	2
163 (488bp)	GTG – GCG	Val – Ala	>75 <100 µg/ml	CDC	1
170 (509bp)	GCC – GTC	Ala – Val	75 µg/ml	CDC	1
180 (538bp)	GTC – ATC	Val – Ile	75 µg/ml	CDC	1

Abbreviations: Ala, Alanine; Arg, Arginine; Asp, Aspartate; bp, base pair; CDC, Centers for Disease Control; Glu, Glutamate; Gly, Glycine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Met, Methionine; MIC, minimum inhibitory concentration; Ser, Serine; Thr, Threonine; Val, Valine.

*This polymorphism had one isolate with an associated MIC of <25 µg/ml and another isolate with an associated MIC of >50 <100 µg/ml.

†This mutation was also identified in literature search.

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Supplemental Table S1: Single-nucleotide polymorphisms detected in the *pncA* gene, by country and resistance phenotype.

SNP location	SNP type	Codon Change	Country and reference	Phenotype linked to SNP	Total Frequency	Sensitive Frequency (%)
-125	C insertion	Upstream	Europe (76)	S	4	100%
-125 & 195	C insertion & C>T	Upstream & Ser65Ser	Europe (76)	S	11	100%
-124 & 195	C deletion & C>T	Upstream & Ser65Ser	Europe (76)	S	1	100%
-33	G insertion	Upstream	Sweden (40)	S	1	100%
-13	G>T	Upstream	Canada (73), Europe (76)	R (73), S (76)	2	50%
-7	T>C	Upstream	Korea (6), USA (35), Thailand (55) , Europe (76)	S (6), N/S (55), R (35,76)	8	12.5%
-3	C insertion	Upstream	Sweden (40,61), Europe (76)	S (40,76), *R/S (61)	4 (r/s – multicentre study)	75%
14	T>C	Ile5Thr	China (72)	S	1	100%
16	A>C	Ile6Leu	Europe (76)	S	128	100%
27	G>C	Val9Val	Peru (14)	S	1	100%
31	A>G	Asn11Asp	China (72)	S	1	100%
35	A>C	Asp12Ala	Cambodia (5), USA (13), China (16,50,72), Japan (20), Korea (7,21), India (27,62), Mexico (28), Peru (34), Canada (73) , Europe (76)	N/S (5,27), R (7,13,16,20,21,28,34,50, 62,73,76), S (72)	28	7%
35	A>G	Asp12Gly	South Africa (3), Europe (76)	R (3), S (76)	11	18%
40	T>G	Cys14Gly	Korea (21), Peru (34), Belgium (45)	S (21,45), R (34)	5	20%
49	G>T	Gly17Cys	Kazakhstan (77)	S	1	100%
62	T>C	Val21Ala	Germany (75), Europe (76)	N/S (75), S (76)	5	80%
82	G>A	Ala28Thr	Netherlands (43)	S	1	100%
92	T>C	Ile31Thr	Thailand (9,55)	*R/S (9), N/S (55)	21	43%
92	T>G	Ile31Ser	Thailand (9), Europe (76)	*R/S (9), R (76)	5	20%
98	A>C	Asp33Ala	Taiwan (19), China (36)	S (19), R (36)	2	50%

104	T>C	Leu35Pro	Thailand (9), India (27), China (36), Japan (49), Spain (63), Europe (76)	R (9,49,63,76), N/S (27), S (36)	6	17%
104	T>G	Leu35Arg	Brazil (24), China (72)	R (24), S (72)	2	50%
114	G>C	Ala38Ala	Europe (76)	S	1	100%
117	2-bp insertion	Frameshift	Korea (6)	S	1	100%
127	C>T	His43Tyr	Netherlands (43)	S	1	100%
134	T>C	Val45Ala	Thailand (9), China (36), Europe (76)	R (9,36), S (76)	3	33%
137	C>T	Ala46Val	Portugal (10), Canada (13,73), USA (13), Korea (13,21), Yemen (23), China (72), Europe (76)	R (10,13,23,73), S (21,72,76)	13	23%
139	A>G	Thr47Ala	Korea (7), Mexico (28), China (33), USA (35,41,71), Sweden (40,67), Europe (76)	R (7,28,33,40,41), S (35,67,71,76)	50	80%
143	A>C	Lys48Thr	Korea (6), Peru (34), Cuba (60), Europe (76)	S (6), R (34,60,76)	10	10%
146	A>C	Asp49Ala	Korea (7,21), Brazil (24), Belgium (45), Japan (49), Europe (76)	R (7,21,45,49,76), *R/S (24)	8	12.5%
147	C>G	Asp49Glu	Brazil (24), Netherlands (43)	S (24,43)	2	100%
188	A>C	Asp63Ala	Mexico (28), Belgium (45), China (51), Russia (65), Singapore (69), Europe (76)	R (28,45,69,76), S (51), N/S (65)	9	11%
191	A>C	Tyr64Ser	Belgium (45), Europe (76)	S (45), *R/S (76)	3	67%
193	T>C	Ser65Pro	China (72)	S	1	100%
195	C>T	Ser65Ser	Iran (2), South Africa (3,66), Japan (20), India (27,53,64), Sweden (40,61,67), USA (41), Tanzania (55), Canada (73) , Europe (76)	R (2), S (3,20,40,41,53,61,64,66, 67,73,76), N/S (27,55)	76	70%
195 & 152	C>T & A>G	Ser65Ser & His51Arg	India (64)	S	1	100%

199	T>C	Ser67Pro	Korea (6,7), Thailand (9), Canada (23), Sweden (40), China (50,51), Canada (73), Europe (76)	R[7,9,23,40,50,51,73,76], S (6)	10	10%
201	TCG insertion	Frameshift	China (72)	S	1	100%
234	C>T	Gly78Gly	China (51)	S	1	100%
245	A>G	His82Arg	USA (13), Brazil (18), China (36,72), Belgium (45), New Zealand (48), Europe (76)	R (13,18,45,48,72,76), *R/S (36)	12	8%
277	G>A	Val93Met	Kazakhstan (77)	S	1	100%
290	G>A	Gly97Asp	South Africa (3), Japan (17), Taiwan (19,31), USA (35), China (36,72), New Zealand (48), Singapore (69), Europe (76)	R (3,17,19,31,35,36,48,69, 76), S (72)	13	8%
304-306	GCG>AGA	Ala102Arg	Europe (76)	S	1	100%
304	G>C	Ala102Pro	Cambodia (5), Europe (76)	N/S (5), S (76)	3	33%
305	C>T	Ala102Val	South Africa (3,66), Russia (4), China (36,72), Europe (76)	R (3,4,36,66) , S (72,76)	10	40%
309	C>G	Tyr103STOP	Russia (4), Korea (7,21), Brazil (18), South Africa (25), Peru (34), USA (35), Europe (76)	R (4,7,18,25,34,35,76), S (21)	17	6%
341	C>T	Thr114Met	South Africa (25)	S	10	100%
347	T>C	Leu116Pro	Peru (34), Italy (42), China (72), Europe (76)	R (34,42,76), *R/S (72)	5	20%
371	G>A	Gly124Asp	Netherlands (43)	S	1	100%
382	G>T	Val128Phe	China (72)	S	1	100%
389	T>C	Val130Ala	Korea (1,21), China (36,50) , Europe (76)	R (1,36,50,76), S (21)	5	20%
401	C>T	Ala134Val	Canada (13), Peru (14), Taiwan (19,31), USA (35), Russia (65), China (72), Europe (76)	R (13,14,19,31,35,76), N/S (65), S (72)	9	11%

403	A>C	Thr135Pro	Korea (1,7,8,21), China (33), Peru (34), USA (35), Belgium (45), Japan (49), Cuba (60), Poland (75), Europe (76)	R (1,7,8,21,33,34,35,49,75, 76), S (45,60)	41	5%
404	C>G	Thr135Ser	Europe (76)	S	1	100%
407	A>G	Asp136Gly	Cambodia (5), Korea (8,21), South Africa (25), China (33,72), Peru (34), Thailand (55), Europe (76)	N/S (5,55), R (8,21,25,33,72), S (34,76)	11	27%
410	A>G	His137Cys	USA (35), Europe (76)	R (35), S (76)	4	50%
419	G>A	Arg140His	Japan (15), South Korea (47)	S (15), N/S (47)	3	66%
419 & 493	G>A & G>A	Arg140His & Ala165Thr	Japan (15)	S	2	100%
419 & 450 & 493	G>A & C>A & G>A	Arg140His & Gly150Gly & Ala165Thr	Japan (15)	S	1	100%
427	G>A	Ala143Thr	Iran (2), Belgium (45), Europe (76)	R (2), S (45,76)	4	75%
460	A>G	Arg154Gly	Korea (1), South Africa (3,66), Russia (4), China (36,72), Europe (76)	R (1,3,4,36,66), S (72), *R/S (76)	8	25%
462	G>T	Arg154Ser	Europe (76)	S	3	100%
464	T>C	Val155Ala	Korea[6,21], Peru (14), China[72]	S (6,72), N/S (14), *R/S (21)	6	66%
470	T>G	Val157Gly	Korea (1), China (72), Europe (76)	R (1,76), S (72)	4	25%
475	C>G	Leu159Val	Europe (76)	S	1	100%
478	A>G	Thr160Ala	China (72), Europe (76)	S (72), R (76)	2	50%
503	C>T	Thr168Ile	Europe (76)	S	2	100%
511	G>A	Ala171Thr	Portugal (10), Bangladesh (11), USA (35), China (72)	R (10,11,35), S (72)	4	25%
512	C>T	Ala171Val	Scotland (11), China (72), Canada (73), Europe (76)	S (11,76), *R/S (72), R (73)	6	67%
521	A>G	Glu174Gly	China (33,54)	R (33), S (54)	2	50%

523	A>G	Met175Val	Japan (20), Brazil (24), China (36), Belgium (45), Thailand (55), Poland (75), Europe (76)	R (20,24,45,75,76), S (36), N/S (55)	14	7%
525	G>A	Met175Ile	Korea (6), China (33), Belgium (45), Brazil (57), Europe (76)	S (6), *R/S (45), R (33,57,76)	15	13%
529	A>C	Thr177Pro	Taiwan (19), China (36)	R (19), S (36)	2	50%
545	T>C	Leu182Ser	Korea (21), France (39), Europe (76)	S (21), R (39,76)	3	33%
545	T>G	Leu182Trp	Europe (76)	S	1	100%
559	T>G	STOP187Gly	India (53), Sweden (61)	R (53), S (61)	2	50%
559	T>C	STOP187Arg	China (72)	S	1	100%

Abbreviations: Ala, Alanine; Arg, Arginine; Asn, Asparagine; Asp, Aspartate; bp, base pair; Cys, Cysteine; Glu, Glutamate; Gln, Glutamine; Gly, Glycine; His, Histidine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Met, Methionine; N/S, not stated; Phe, Phenylalanine; Pro, Proline; R, resistant; S, susceptible; Ser, Serine; SNP, single-nucleotide polymorphism; Thr, Threonine; Trp, Tryptophan; Tyr, Tyrosine; Val, Valine.

* Article reported one isolate sensitive and one isolate resistant.

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Chapter 5

Fitness effect of *pncA* mutations on the growth rate of *Mycobacterium tuberculosis*

My contribution:	Generation of PZA *In Vitro* mutants
	Planning of experimental work
	Genotyping of all strains
	Culturing strains and performing drug susceptibility tests
	Interpretation of results and data

Abstract

Background

PZA is an integral component of the current standardized treatment regimen recommended by the World Health Organization for the treatment of TB, because of its ability to shorten treatment to 6 months when combined with isoniazid, rifampicin and ethambutol. Deleterious mutations in *pncA* gene are the primary mechanism of resistance to PZA. Extensive sequencing studies of *pncA* have revealed that mutations, small insertions and deletions occur across the entire length of the gene. The impact of these mutations on the growth characteristics of *Mycobacterium tuberculosis* has not been investigated, which has resulted in contrasting views emerging in the literature. This study aimed to investigate the *in vitro* growth rates of isolates harbouring different *pncA* mutations with the aim to determine the impact on these mutations on growth rate as a surrogate for fitness.

Methods

Growth rates measured using BD BACTEC MGIT 960 time to positivity (TTP) were compared between isolates with and without *pncA* mutations (single nucleotide polymorphisms (SNPs) and large deletions) matched for resistance to other anti-TB drugs where possible. A one way ANOVA was used to determine significance.

Results

No difference was observed in the TTP in MGIT media when comparing wild type and strains harbouring *pncA* SNPs, thereby suggesting that *pncA* SNPs do not have a significant fitness cost. However, strains harbouring large deletions encompassing the *pncA* gene showed a significant growth deficit (p-value: <0.001) *in vitro*.

Conclusion

This study suggests that SNPs in the *pncA* do not influence on the growth of *M. tuberculosis*. In contrast, deletions encompassing the *pncA* gene retarded the *in vitro* growth rate. However, this growth deficit did not prevent these strains from transmitting in the community. This differs from

previous studies which have suggested that *in vitro* growth rate is a surrogate for fitness cost which reduces the ability of *M. tuberculosis* to transmit.

Introduction

Several efforts have been made to determine the genetic constituents required for growth and survival of *Mycobacterium tuberculosis* by means of screening transposon site hybridization (TraSH) libraries (1-3). These studies reveal that a large proportion of genes are non-essential for growth and are able to harbour mutations, while still allowing survival of the pathogen. Deletion of a non-essential gene involved in converting a pro-drug to an active drug is an alternative mechanism of drug resistance. One such example is the *pncA* gene which has previously been reported to undergo deletion in clinical strains (4-6). *pncA* encodes pyrazinamidase (PZase), an enzyme that converts pyrazinamide (PZA) to its active form pyrazinoic acid (POA) (7). PZA is an integral component of the current standardized treatment regimen recommended by the World Health Organization for the treatment of TB, because of the shortening of treatment to 6 months when combined with isoniazid, rifampicin and ethambutol (8). Deleterious mutations in *pncA* gene are the primary mechanism of resistance to PZA (9). Extensive sequencing studies of *pncA* have revealed that mutations, small insertions and deletions occur across the entire length of the gene (10-12).

Recent studies have demonstrated that mutations linked with drug resistance could result in a fitness cost for the organism (13-16). Most studies have focused on determining the impact of mutations in the *rpoB* and *rrs* genes (14, 15, 17). These studies have highlighted how different mutations have different effects on *in vitro* growth. Accordingly these mutations have been classified as either being high or low cost mutations. Importantly, compensatory mutations have also been identified which ameliorate the fitness cost associated of the *rpoB* or *rrs* mutations (17, 18). To date no studies have reported whether mutations in *pncA* have a fitness cost, although the authors of a recent systematic review suggested that PZA resistance has a low fitness cost due to the observed clustering of PZA resistant strains (19). This contrasts with the view of den Hertog *et al* who suggested that *pncA* mutations induce a fitness cost which may impair the organism's ability to transmit (20). Their opinion was based on the lack of extensive clustering of *pncA* mutations causing PZA resistance.

This study aimed to investigate the *in vitro* growth rates of *M. tuberculosis* isolates harbouring different *pncA* mutations (SNPs and large deletions) with the view to determine the impact on these mutations on growth as a surrogate for fitness.

Methods

Pyrazinamide resistant *M. tuberculosis* isolates

In vitro selected PZA resistant mutants

Spontaneous PZA resistant clones were selected on 7H10 media containing 100 µg/ml PZA (21) using a pan-susceptible *M. tuberculosis* Beijing genotype progenitor strain according to the method described by Morlock *et al* (22). Colonies were picked and sub-cultured in 5 ml OADC-enriched 7H9 medium. A total of 74 clones were selected for further analysis. All clones were confirmed to be resistant to PZA using the PZA DST BACTEC Mycobacteria Growth Indicator Tube (MGIT) system (Becton Dickinson, Sparks, MD).

Clinical PZA resistant strains

Twenty-three clinical *M. tuberculosis* isolates harbouring large *pncA* deletions were identified in the local database of *M. tuberculosis* whole genome sequences (personal communication EM Streicher). In addition, a selection of phenotypically PZA resistant (n = 23) and susceptible clinical isolates (n = 23) were selected. These isolates were selected based on their drug resistance profile to ensure that they matched the isolates with large *pncA* deletions. The mechanism conferring PZA resistance was determined using Sanger sequencing of the *pncA* gene (23).

pncA Sanger sequencing

Amplification and sequencing of the *pncA* gene was performed as described by Streicher *et al* (23). Briefly, the *pncA* gene was amplified with primers JpncAs Forward and JpncAs Reverse (Table 1) and a standard PCR mix of 1X Buffer, 0.75 µM of each primer, 0.8 mM dNTPs and 0.125 µl (0.625 U) Taq polymerase (Qiagen, Germany). These primers were designed to amplify a wild type (WT) 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. The amplification protocol consisted of an initial activation step of 95°C for 15 minutes, followed by 50 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute and a final elongation step of 10 minutes at 72°C. PCR products were sequenced in both directions with the JpncAs Forward and JpncAs Reverse primers.

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. Isolates with *pncA* deletions failed to amplify. The DNA sequencing reaction was done on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA. Sequences were analysed on the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html) with H37Rv as the reference strain.

The boundaries of the deletions were determined by Sanger sequencing. Briefly, PCR primers were designed to span the deleted region identified by whole genome sequencing (Table 1). Each boundary was amplified with the corresponding primers using a standard PCR mix containing 1X Buffer, 0.75 µM of each primer, 0.8 mM dNTPs and 0.125 µl (0.625 U) Taq polymerase (Qiagen, Germany). The amplification protocol consisted of an initial activation step of 95°C for 15 minutes, followed by 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute and a final elongation step of 10 minutes at 72°C. Amplification and band size was confirmed by gel electrophoresis (data not shown). Each amplicon was Sanger sequenced using the corresponding primers (Table 1).

PZA susceptibility testing

All isolates (clinical and *in vitro* mutants) were assessed for PZA susceptibility using the non-radiometric BACTEC MGIT 960 method as per the manufactures instructions (BD Diagnostic Systems, NJ, USA). This system makes use of modified test media which supports the growth of mycobacteria at a pH of 5.9. In order to distinguish between resistant and susceptible isolates a critical concentration of 100 µg/ml PZA was utilized. Briefly an isolate was cultured in BACTEC MGIT using OADC enriched media; this was considered the starter culture. Two days after the starter culture had reached the growth threshold (400 growth units), the PZA DST was prepared. A 1:10 dilution of the starter culture was made in a sterile saline solution. Two PZA MGIT tubes are prepared: the first is the growth control tube which was supplemented with 800 µl of enriched media (pH adjusted) and 500 µl of the diluted starter culture. The second tube is the PZA tube which was supplemented with 800 µl of enriched media (pH adjusted), 100 µl of PZA (to a final concentration of 100 µg/ml), and 500 µl of undiluted started culture. The MGIT tubes were logged using the EpiCentre™ system (BD

BACTEC) and tubes were loaded into the BD BACTEC MGIT machine. Samples results were read continuously for 20 days. If no growth has occurred in the PZA containing tube the sample was considered to be PZA susceptible and if grow had occurred in the PZA tube prior to the growth control tube reaching the growth threshold (400 growth units) the sample was considered PZA resistant. Due to the nature of DST a standardization of inoculum size was utilized.

PZA minimum inhibitory concentration (MIC) testing

Representatives of the *pncA* large deletion isolates (where possible 2 isolates of each unique deletion) were selected for PZA MIC testing in order to determine level of resistance. Three concentrations of PZA (300 µg/ml, 600 µg/ml and 900 µg/ml) were tested using the PZA susceptibility testing method described above.

Growth rates (time to positivity)

Time to positivity (TTP) was determined by measuring the number of hours that it took for the PZA DST growth control of each isolate to reach 400 Growth Units as measured by EpiCentre™ system (BD BACTEC) software. The TTP data was analyzed using GraphPad Prism. In order to determine the significance between TTP/*in vitro* growth rates a one way ANOVA was utilized to determine p-values.

Table 1. Primers for amplification of the *pncA* gene and large deletion junctions.

JpncAs Forward	5′ – GGCGTCATGGACCCTATA – 3′
JpncAs Reverse	5′ – GTGAACAACCCGACCCAG – 3′
881_del_For	5′ – CGATGGAACGTGATATCCCGC – 3′
881_del_Rev	5′ – CCTTTTCTGTCAGTGGCCGG – 3′
2181_del_For	5′ – CGATGGAACGTGATATCCCGC – 3′
2181_del_Rev	5′ – ATCCGTCATACAGGCCACCG – 3′
298_del_For	5′ – CGATGGAACGTGATATCCCGC – 3′
298_del_Rev	5′ – TGACCACTTCTCCGGCACAC – 3′
3667_del_For	5′ – GGCCCGTACCTTGATATCGGG – 3′
3667_del_Rev	5′ – ATCCGTCATACAGGCCACCG – 3′
3548_del_For	5′ – GGCCCGTACCTTGATATCGGG – 3′
3548_del_Rev	5′ – ATCCGTCATACAGGCCACCG – 3′
17133_del_For	5′ – GATCCGTCGATGCCCACCAA – 3′
17133_del_Rev	5′ – CATGGGACGGGTTTTCGCTG – 3′

Results

A total of 74 colonies were picked from the PZA 100 µg/ml 7H10 plates, termed *in vitro* (*IV*) mutants. Five of the 74 (6.8%) were contaminated while the remaining 69 (93.2%) were successfully amplified and the *pncA* gene was sequenced as well as having a PZA DST result (Supp Table 1). Twenty-four (34.8%) *IV* mutants which showed a WT genotype for *pncA* were found to be resistant to PZA at 100 µg/ml. The remaining 45 (65.2%) *IV* mutants were found to have mutations in the *pncA* gene, spanning the entire length of the gene. A total of 29 different *pncA* mutations (which have been described in clinical isolates previously), including single nucleotide polymorphisms (SNPs); insertions; deletions and double SNPs were identified (Supp Table 1). All *IV* mutants with *pncA* mutations were confirmed to be resistant to PZA at 100 µg/ml.

Twenty three clinical isolates harbouring large deletions encompassing the *pncA* gene were selected, termed Δ *pncA*. These deletions ranged in size; 299 bp, 881 bp, 2181 bp, 3455 bp, 3668 bp and 17133 bp. These deletions were initially detected by means of whole genome sequencing (WGS) and the boundaries were confirmed using Sanger sequencing of the deletion junctions. Details of the deletions are shown in Supplemental Table 2 and visually illustrated in Figure 1. All the *pncA* deletions had a PZA MIC of >900 µg/ml (Supp Table 2).

A total of 46 clinical isolates were selected of which 23 (Supp Table 4) were susceptible to PZA (termed WT *pncA*) and 23 (Supp Table 3) were resistant to PZA (termed MUT *pncA*). The PZA susceptible isolates were all confirmed to have a *pncA* wild type genotype and the PZA resistant isolates were all confirmed to have a mutation in the *pncA* gene. The 46 clinical isolates (XDR-TB) were matched according to drug resistance profile with the strains harbouring *pncA* large deletions (XDR-TB). The resistant clinical isolates had 23 different mutations in the *pncA* gene; 21 non-synonymous SNPs and 2 insertions (Supp Table 3).

To determine the influence of mutation in the *pncA* gene on TTP the following comparisons were made: 1) WT *pncA* vs *IV* mutant's; 2) *IV* mutant's vs MUT *pncA*; 3) *IV* mutant's vs Δ *pncA*; 4) MUT *pncA* vs Δ *pncA*, 5) WT *pncA* vs MUT *pncA*, 6) WT *pncA* vs Δ *pncA*. Figure 2 shows a graphic representation of the difference in TTP for each of the groups. The mean TTP for each group is as follows, 203 hours for the Δ *pncA* group, 143 hours for the MUT *pncA* group, 129 hours for the *IV* mutant's group, and 121 hours for the WT *pncA*. A significant difference (p value: <0.001) was observed between the growth rate of the Δ *pncA* isolates were compared to the growth rates of the MUT *pncA* isolates, *IV* mutant's and the WT *pncA* isolates. However, no significant difference was observed when the growth rates of the MUT *pncA* isolates and *IV* mutants were compared, or when the WT *pncA* isolates and MUT *pncA* were compared or when the WT *pncA* isolates and the *IV* mutant's were compared.

Discussion

Using TTP in combination with a standardised inoculum, we are unable to show a significant difference in growth rate between WT strains and strains harbouring *pncA* SNPs or small indels or *IV*

mutants. This suggests that the loss of pyrazinamidase activity does not influence *in vitro* growth rate which is in line with previous findings that have demonstrated that the *pncA* gene is not essential for *in vitro* growth (11, 24). Furthermore, molecular epidemiological studies conducted in South Africa (25, 26), USA (27) Belgium (19), and Russia (28) confirm that strains harbouring *pncA* mutations retain their ability to transmit. Together this suggests that *pncA* mutations do not have a fitness cost. These findings support the observation that *pncA* mutations have a low fitness cost according to Stoffels *et al* (19). This is in contrast to the views of den Hertog *et al* who suggested that PZA resistance induces a fitness cost that impairs *M. tuberculosis* transmission (20).

We identified a significant difference in the *in vitro* growth rate between strains harbouring deletions encompassing the *pncA* gene. This growth deficit was not dependent on the size of the deletion, suggesting that altering the spatial arrangement of the genes surrounding the *pncA* gene has an influence of *in vitro* growth rate. The slower growth rate of these strains suggests that they may have an impaired ability to transmit as has been suggested for other high fitness cost drug resistance causing mutations (13). However, two clusters (more than one isolate with the same deletion boundary) were identified among the 23 isolates with large *pncA* deletions. Interestingly, the 881 bp *pncA* deletion was present in isolates from 17 patients thereby confirming the transmissibility of this strain. Transmission of these strains was further supported by the fact these deletions had identical boundaries and were of the Beijing genotype.

We acknowledge that this study has certain limitations. Firstly, we cannot exclude the possibility that other mutational events could have impacted on the *in vitro* growth rate in isolates harbouring large *pncA* deletions. This is unlikely given that 17 of the 23 isolates were from the same genetic background. Secondly, the growth rates were not compared in the same tube (a competition assay). Thirdly, the MGIT TTP is a relatively crude assessment of growth. The latter limitations could be addressed using molecular cloning to generate *pncA* deletions into the same progenitor strain. The competition assay would be a more accurate assessment of fitness cost.

In conclusion this study suggested that SNPs and small indels do not influence *in vitro* growth rate. In contrast, large *pncA* deletions significantly slow the *in vitro* growth rate but do not prevent the transmission of these strains. This suggests a disconnect between *in vitro* growth rate and *in vivo*

transmission. This differs from previous studies which have suggested that *in vitro* growth rate is a surrogate for fitness cost and the inability to transmit (14, 15, 29-31). This may be related to the difference in essentiality of the genes being tested.

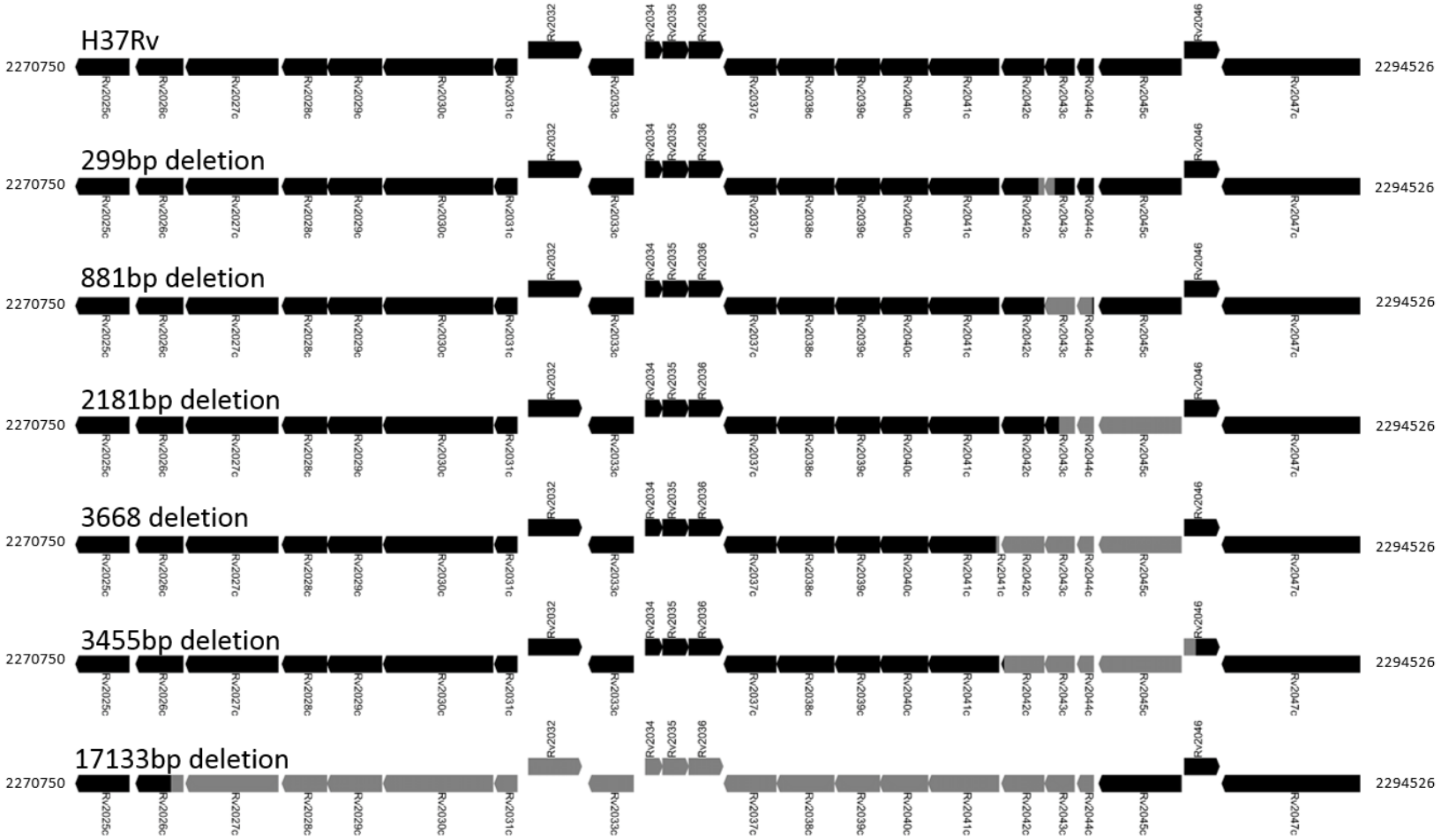

Figure 1. Visual illustration of large deletions encompassing the *pncA* gene (*Rv2043c*) in clinical isolates of *M. tuberculosis.* Light grey areas indicate deletions and black areas indicate intact genomic regions relative to H37Rv. Genes are indicated with Rv numbers and the region included spans from nucleotides 2270750 to 2294526.

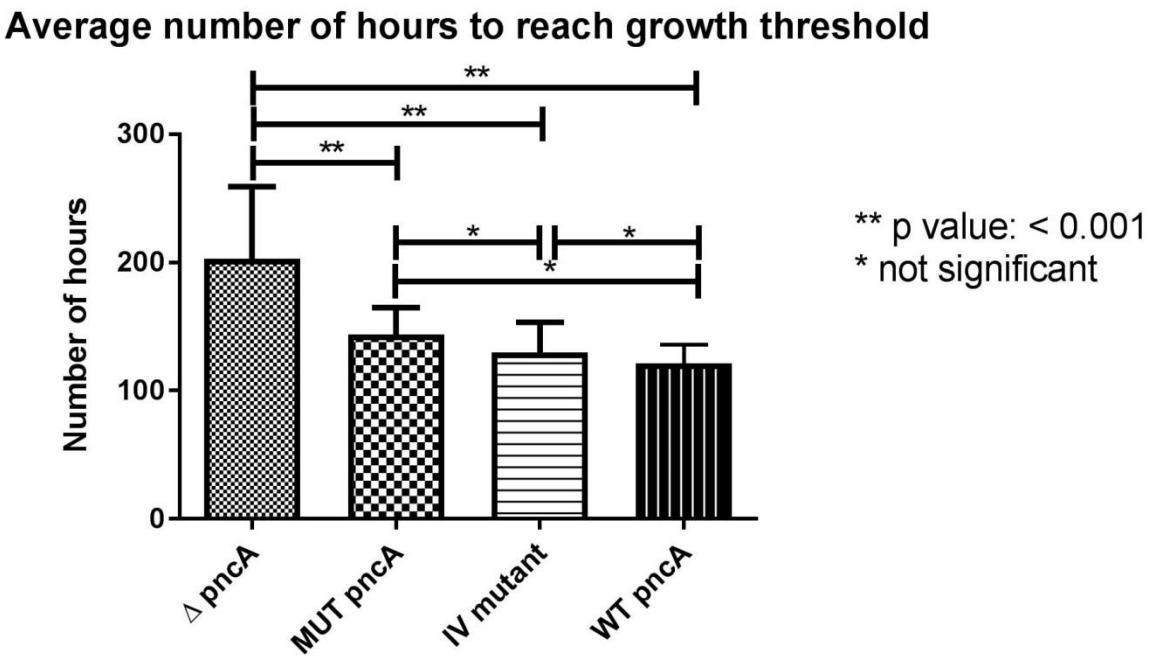

Figure 2. Average number of days to reach growth threshold of 400 growth units for Δ *pncA*, MUT *pncA*, *IV* mutant, and WT *pncA*, respectively. A one way ANOVA test was utilized.

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Supplemental Table 1. *IV mutant* spontaneous *M. tuberculosis* isolates.

Sample	***pncA* SNP**	**Phenotypic PZA DST**	**Time to positivity (hours)**
1	Gln9Lys	Resistant	122
2	Tyr99STOP	Resistant	147
3	Tyr34STOP	Resistant	176
4	Tyr34STOP	Resistant	148
5	Tyr34STOP	Resistant	125
6	Tyr34STOP	Resistant	160
7	His57Asn	Resistant	149
8	CONTAM	CONTAM	CONTAM
9	Trp68Gly	Resistant	108
10	Tyr34STOP + Tyr99STOP	Resistant	155
11	Trp119Cys	Resistant	159
12	Thr160Lys	Resistant	97
13	Thr160Lys	Resistant	122
14	Ser18STOP	Resistant	132
15	Val7Phe	Resistant	114
16	Tyr99STOP	Resistant	119
17	INS 146 (T)	Resistant	156
18	Gln10Arg + Glu91STOP	Resistant	118
19	INS 100 (C)	Resistant	100
20	Glu127STOP	Resistant	125
21	Ala102Val	Resistant	108
22	Ser18STOP	Resistant	148
23	Tyr34STOP	Resistant	97

24	Trp68Leu + Ala146Thr	Resistant	99
25	WT	Resistant	148
26	CONTAM	CONTAM	CONTAM
27	DEL 128 (8bp)	Resistant	121
28	Cys14STOP	Resistant	152
29	Tyr103STOP + Val139Met	Resistant	156
30	Ser18STOP	Resistant	125
31	Ala102Val	Resistant	162
32	Tyr99STOP	Resistant	160
33	INS 131 (G)	Resistant	122
34	INS 131 (G)	Resistant	151
35	Gln10Lys	Resistant	131
36	WT	Resistant	186
37	WT	Resistant	114
38	WT	Resistant	165
39	WT	Resistant	132
40	Tyr34STOP	Resistant	124
41	WT	Resistant	100
42	Tyr34STOP	Resistant	121
43	WT	Resistant	122
44	Tyr99STOP	Resistant	132
45	WT	Resistant	123
46	Asp12Asn	Resistant	106
47	WT	Resistant	108
48	WT	Resistant	143
49	Ala26Asp	Resistant	142

50	Ala144Pro	Resistant	149
51	Trp68STOP	Resistant	146
52	INS 134 (A)	Resistant	132
53	WT	Resistant	119
54	WT	Resistant	98
55	WT	Resistant	124
56	WT	Resistant	151
57	Gln10His	Resistant	155
58	WT	Resistant	122
59	WT	Resistant	128
60	WT	Resistant	122
61	WT	Resistant	100
62	WT	Resistant	132
63	WT	Resistant	114
64	INS 70 (G)	Resistant	143
65	CONTAM	CONTAM	CONTAM
66	CONTAM	CONTAM	CONTAM
67	CONTAM	CONTAM	CONTAM
68	His49Asn	Resistant	127
69	Tyr99STOP	Resistant	122
70	WT	Resistant	123
71	WT	Resistant	180
72	WT	Resistant	166
73	WT	Resistant	124
74	Ile45Ser	Resistant	145

Supplemental Table 2. Δ *pncA* clinical *M. tuberculosis* isolates.

Sample	*pncA* large deletion	Phenotypic PZA DST	Time to positivity (hours)	Drug resistance classification
1	17133 bp	Resistant (MIC >900)	171	XDR-TB
2	3668 bp	Resistant (MIC >900)	194	MDR-TB
3	3455 bp	Resistant (MIC >900)	204	MDR-TB
4	881 bp	Resistant	239	XDR-TB
5	881 bp	Resistant	169	XDR-TB
6	881 bp	Resistant	195	XDR-TB
7	881 bp	Resistant	160	XDR-TB
8	881 bp	Resistant	173	XDR-TB
9	881 bp	Resistant	234	XDR-TB
10	881 bp	Resistant	152	XDR-TB
11	881 bp	Resistant	251	XDR-TB
12	881 bp	Resistant	317	XDR-TB
13	881 bp	Resistant	376	XDR-TB
14	881 bp	Resistant	149	XDR-TB
15	881 bp	Resistant	226	XDR-TB
16	881 bp	Resistant	160	XDR-TB
17	881 bp	Resistant	165	XDR-TB
18	881 bp	Resistant	182	XDR-TB
19	881 bp	Resistant (MIC >900)	218	XDR-TB
20	881 bp	Resistant (MIC >900)	152	XDR-TB
21	2181 bp	Resistant (MIC >900)	146	XDR-TB
22	299 bp	Resistant (MIC >900)	199	MDR-TB
23	299 bp	Resistant (MIC >900)	234	MDR-TB

Supplemental Table 3. MUT *pncA* clinical *M. tuberculosis* isolates.

Sample	*pncA* SNP	Phenotypic PZA DST	Time to positivity (hours)	Drug resistance classification
1	Cys14Arg	Resistant	148	MDR-TB
2	Leu120Pro	Resistant	154	XDR-TB
3	Ile131Ser	Resistant	150	XDR-TB
4	Gly102Val	Resistant	143	XDR-TB
5	INS: Codon 173 (G)	Resistant	163	XDR-TB
6	INS: Codon 12 (G)	Resistant	147	XDR-TB
7	Val139Ala	Resistant	146	XDR-TB
8	Tyr103STOP	Resistant	135	XDR-TB
9	Asn118Ser	Resistant	152	MDR-TB
10	Glyc97Asp	Resistant	97	XDR-TB
11	Ala134Val	Resistant	131	XDR-TB
12	Ser59Pro	Resistant	122	XDR-TB
13	His71Tyr	Resistant	127	XDR-TB
14	Ser179Arg	Resistant	97	MDR-TB
15	Ser185Thr	Resistant	179	XDR-TB
16	Val9Leu	Resistant	149	XDR-TB
17	Ala146Thr	Resistant	190	XDR-TB
18	Tyr103His	Resistant	158	XDR-TB
19	Ser164Pro	Resistant	149	XDR-TB
20	Met1Thr	Resistant	151	XDR-TB
21	Gly132Ala	Resistant	143	XDR-TB
22	Gly97Asp	Resistant	146	XDR-TB
23	Asp8Asn	Resistant	130	XDR-TB

Supplemental Table 4. WT *pncA* clinical *M. tuberculosis* isolates.

Sample	*pncA* SNP	Phenotypic PZA DST	Time to positivity (hours)	Drug resistance classification
1	Wild type	Susceptible	132	XDR-TB
2	Wild type	Susceptible	145	XDR-TB
3	Wild type	Susceptible	128	XDR-TB
4	Wild type	Susceptible	135	MDR-TB
5	Wild type	Susceptible	107	MDR-TB
6	Wild type	Susceptible	125	XDR-TB
7	Wild type	Susceptible	119	MDR-TB
8	Wild type	Susceptible	112	MDR-TB
9	Wild type	Susceptible	122	MDR-TB
10	Wild type	Susceptible	127	XDR-TB
11	Wild type	Susceptible	97	MDR-TB
12	Wild type	Susceptible	104	XDR-TB
13	Wild type	Susceptible	118	XDR-TB
14	Wild type	Susceptible	100	MDR-TB
15	Wild type	Susceptible	103	XDR-TB
16	Wild type	Susceptible	153	XDR-TB
17	Wild type	Susceptible	114	XDR-TB
18	Wild type	Susceptible	104	MDR-TB
19	Wild type	Susceptible	130	XDR-TB
20	Wild type	Susceptible	121	XDR-TB
21	Wild type	Susceptible	122	XDR-TB
22	Wild type	Susceptible	136	MDR-TB
23	Wild type	Susceptible	134	XDR-TB

Chapter 6

Virtual sequencing of the entire *pncA* gene target in a single tube using LATE-PCR and Lights-On/Lights-Off probes to predict PZA susceptibility

My contribution:	Design of probes
	Genotyping of all South African strains
	Planning of experimental work
	Performing the assay
	Interpretation of results and data
	Chapter part of larger collaboration which will be published
	Assisting with current draft manuscript for publication

Abstract

Background

The anti-TB drug pyrazinamide (PZA) is an integral component of the standardized TB treatment regimen recommended by the WHO. The continued inclusion of PZA in novel anti-TB treatment regimens is also largely due to its unique synergistic properties with new TB drugs, namely Protomanid and Bedaquiline. Unfortunately, drug susceptible testing (DST) for PZA is rarely performed, due to associated limitations inherent to DST. This is a major problem because growing evidence supports the fact that a great many mutations in the *pncA* gene confer resistance to PZA and that only a wild type *pncA* genotype correlates with PZA susceptibility. A rapid high resolution method for analysing the sequence of the *pncA* gene is therefore urgently needed.

Methods

LATE-PCR based technique in combination with fluorescence probes to identify mutations along the entire length of the *pncA* gene. Following amplification, the temperature of the reaction is lowered to allow the 34 Lights-On/Lights-Off fluorescent probes to cover the single-stranded DNA templates. A fluorescent signature reveals the presence/absence of mutations, which are all distinguished from a highly reproducible wild-type fluorescent signature.

Results

The assay has been tested against 414 purified DNA isolates. These isolates comprise 184 (44.4%) *pncA* wild type and 230 (55.6%) *pncA* mutant strains. Among these isolates 179 (98.4%) were correctly genotyped as *pncA* wild type and 229 (98.7%) were correctly genotyped as *pncA* mutant. 3 wild type isolates were incorrectly called as mutant and 3 isolates with mutations were incorrectly called as wild type. This results in a sensitivity of 98.7% (95% CI: 97.3 – 100) and specificity of 98.4% (95% CI: 96.5 – 100).

Conclusion

We have successfully designed and tested a single closed-tube assay that is able to identify and distinguish the wild type *pncA* gene from strains with any genetic alterations and provides an accurate and rapid method to predict a PZA susceptible strain. Additional refinements will improve identification of the rare synonymous mutations and the few non-synonymous mutations in *pncA* that were not correctly scored as PZA resistance. This virtual sequencing method is a promising advance in novel diagnostics for PZA.

Introduction

Mycobacterium tuberculosis is a pathogen which causes tuberculosis (TB), an airborne infectious disease which is curable if diagnosed and treated. The current burden of TB on the health authorities can in part be attributed to drug resistance (DR). It is important to control and reduce the rates of DR-TB as it places a huge financial and public health burden on national TB control programmes across the globe (1).

The anti-TB drug pyrazinamide (PZA) is an integral component of the standardized TB treatment regimen for drug susceptible and drug resistant TB recommended by the WHO (2, 3). PZA will remain an important anti-TB drug in the future due to inclusion in the latest WHO update on MDR-TB treatment guidelines (3). The continued inclusion of PZA in novel anti-TB treatment regimens is also largely due to its unique synergistic properties with new TB drugs, namely Protomanid and Bedaquiline (4, 5). It is thus important to be able to detect resistance to PZA so that the patient's treatment is not compromised. Unfortunately, drug susceptibility testing (DST) for PZA is rarely performed, due to pH and inoculum size limitations inherent to DST (6-8). For this reason, the WHO has not approved a standardized culture-based method for PZA DST due to the complexity and inconsistency of the currently available culture-based tests (2, 9-11).

This has led to many researchers investigating the genotypic relationship to phenotypic PZA resistance. Numerous studies have demonstrated that mutations in *pncA* are the primary mechanism of resistance to PZA (12-14). It is hypothesized that mutations in the *pncA* gene substantially decreases PZase activity, thereby limiting the conversion of PZA to the active form of pyrazinoic acid (POA) in *M. tuberculosis* (15). Extensive DNA sequencing studies of the *pncA* gene have revealed that mutations and/or polymorphisms occur across the entire length of the gene, making it a complex target for the development of a genetic-based DST to identify resistance causing mutations (16-19). A recent systematic review identified over 600 mutations in the *pncA* gene (20). This implies that sequencing the entire *pncA* gene is essential in order to capture all the possible mutations and to differentiate between those that cause and those that do not cause. Recent studies have also suggested that PZA resistance may be conferred via an alternative mechanism(s), namely the *rspA* and *panD*

genes (21, 22). However, due to the lack of convincing evidence of their involvement in PZA resistance, *pncA* remains the primary gene conferring resistance (23, 24).

Culture-based phenotypic DST is time consuming and requires specialized laboratories and laboratory equipment. Sanger DNA sequencing of *pncA* is attractive alternative DST (8, 12-14). While Sanger sequencing significantly improves time to identifying resistance, a rapid point of care diagnostic similar to GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA) would be the ideal solution (25). The most commonly used genetic method to identify changes in the *pncA* gene is standard polymerase chain reaction (PCR) of the target region (*pncA*) and subsequent Sanger sequencing of the amplicon. Other, more expensive genetic methods include deep sequencing and whole genome sequencing (26).

Linear-after-the-exponential (LATE)-PCR is a form of non-symmetric PCR that efficiently generates single-stranded DNA by use of a combination of a Limiting Primer (^{L}) and Excess Primer (^{X}) with different initial $_{[0]}$ concentrations and different melting temperatures (Tm) and fit the formula: $Tm^{L}_{[0]}$-$Tm^{X}_{[0]} \geq 0$. The initial amplification step involves exponential amplification. However, when the Limiting Primer runs out the kinetics switch to linear amplification of the Excess Primer strand only. Thus, LATE-PCR generates a large number of single strands of the target gene. This study makes use of LATE-PCR coupled with Lights-On/Lights-Off probes to identify changes in a desired target region (27-29) and to relate these finding to Sanger sequencing results as well as culture-based phenotypic susceptibility data.

Methods

Clinical isolate selection

A convenience sample set of 414 clinical *M. tuberculosis* isolates (both PZA susceptible and PZA resistance genotypes) were selected for testing the diagnostic utility of LATE-PCR in combination with Lights-On/Lights-Off probes for the detection of *pncA* mutations conferring PZA resistance. These samples were selected from a culture bank of isolates collected from patient's resident in four provinces in South Africa; Free State; Eastern Cape; Gauteng and the Western Cape. This study was approved by the Health Ethics Research Committee of Stellenbosch University (Ethics number: N12/01/001).

DNA extraction

DNA was purified from each isolate as per the procedure as described by Warren *et al* (30). Briefly, each clinical isolate of *M. tuberculosis* was inoculated under biosafety level 3 conditions onto solid 7H10 media and incubated at 37°C for 4-6 weeks. The plates were then transferred to an oven at 80°C for 1 hour to ensure heat killing of mycobacteria. 30 ml of extraction buffer (5% sodium glutamate, 50 mM Tris-HCl [pH 7.4], and 25 mM EDTA) was added to each plate in a biosafety class 2 laminar flow hood, and the colonies were gently scraped from the solid medium with a disposable loop. The liquid suspension from the plates was then transferred to a sterile 50 ml polypropylene tube containing approximately 20 glass beads (diameter, 4 mm) and vigorously vortexed to disrupt all colonies. Lysozyme (25 mg; Roche, Germany) and RNase A (50 g; Roche, Germany) were added, and the suspension was incubated after gentle mixing for 2 hours at 37°C. Thereafter, 600 µl of 10x proteinase K buffer (5% sodium dodecyl sulfate, 100 mM Tris-HCl [pH 7.8], 50 mM EDTA) and 1.5 mg of proteinase K (Roche, Germany) were added and the suspension was incubated at 45°C for 16 hours. An equal volume of phenol-chloroform-isoamyl alcohol (25/24/1) was then added and intermittently mixed over a period of 2 hours at room temperature. Following centrifugation at 3,000 x g for 20 minutes, the resultant aqueous phase was re-extracted with an equal volume of chloroform-isoamyl alcohol (24/1) and centrifuged as described above. The resultant DNA was then precipitated with the addition of 3 M sodium acetate (pH 5.2) (600 µl) and an equal volume of isopropanol and immediately collected on a fine glass rod. The DNA was washed in 70% ethanol and allowed to air dry at room temperature. The purified DNA was re-dissolved in 300 µl TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Genotypic characterisation

The *pncA* gene was Sanger sequenced for all samples according to the method of Streicher *et al* (25). Briefly the *pncA* gene was amplified using the primers shown in Table 1. 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 (purified DNA), 0.75 µM each PCR primer, 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 minutes, followed by 50 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute and a final elongation step of 10 minutes 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 clean-up 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 (Table 1). The DNA sequencing reaction was done on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following program: 5 minutes at 94°C, followed by 25 cycles of 94°C for 10 seconds, 55°C for 10 seconds, and 60°C for 4 minutes, 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 analysed on the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html) with H37Rv as the reference strain.

LATE-PCR

LATE-PCR primers (Table 1) were designed to amplify a 685 bp amplicon encompassing the entire coding region of the *pncA* gene, including 37 bp of the upstream sequence and 24 bp of the downstream sequence. Lights-On/lights-Off probes were designed to cover the amplicon, according to Rice *et al* protocol (28). Briefly, probes of between 20-26 bp were designed to complement the single stranded DNA generated by the LATE-PCR of the selected target, *pncA*. Each Lights-On probe is labelled with a fluorophore, a molecular beacon with a two base pair self-complementary stem. A Lights-Off probe is labelled with only a quencher moiety, such as a black hole quencher. Each Lights-Off probe serves to absorb energy from the fluorophore of its adjacent Lights-On probe, when both are bound to a target. As the signal from each Lights-On probe is extinguished, multiple pairs of

probes of the same colour or different colours can be used to analyse sequences several hundred nucleotides long. These probes are grouped into different fluorophore colours as well as a temperature range from high to low (Figure 1). Some probes included nucleotide mismatches to their target sequences to adjust the probe melting temperature. The probe Tm and binding were checked on Visual-OMP software (DNA Software Inc., Ann Arbor, MI, USA). Each fluorescent contour was transformed into its first derivative curve, which we refer to as a fluorescent signature.

Each reaction mixture (25 µl) contained 1 to 2.5 µl of template DNA (purified DNA), 10 µM of the pncA_LP3 and 100 µM of the pncA_XP3 PCR primers (Table 1), 10× PCR buffer, 50 mM magnesium, 10 µM of prime safe, 10 mM deoxynucleoside triphosphates, 10 µM of each of the ON probes and 200 µM of each of the OFF probes and 0.125 µl (1.5 U) of Taq polymerase (Invitrogen, United States of America). All reagents were added in a PCR preparation room, free of any DNA contamination. The reagent mix was then taken to another clean room where the DNA for each sample was added under sterile conditions and the tube sealed. This was done in order to prevent any cross contamination of the sample and to minimize the risk of PCR contamination. Water was included as a negative control, while H37Rv DNA was included as a positive control. The amplification protocol consisted of 60 cycles of 97°C for 7 seconds, followed by 77°C for 1 minute; this was followed by a final elongation step of 75°C for 10 minutes. Following amplification (generation of single stranded DNA), the temperature of the reaction was lowered to 25^{o}C for 10 minutes. This results in the coating of the single-stranded DNA templates with 34 Lights-On/Lights-Off fluorescent probes labeled in three colors. The temperature is then increased from 25^{o}C to 95^{o}C in a step wise gradient, 1°C for 30 seconds to allow the machine to measure the fluorescence; the probes are melted off of the target and the resulting fluorescent signature. Each fluorescent signature was compared to the signature of a pan-susceptible strain (H37Rv). Deviations in the signature pattern were interpreted to reflect the presence mutations in the *pncA* gene as they would alter the melting temperature of the complementary probe.

If any discordance between the LATE-PCR and Sanger sequencing results were observed, the LATE-PCR was repeated and the single stranded products were subsequently Sanger sequenced using the GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) as described above.

Statistical analysis

The sensitivity and specificity of the DNA sequencing method were determined with Statistica 7.1.

Table 1. Primers for amplification of the *pncA* gene.

JpncAs Forward	5′ – GGCGTCATGGACCCTATA – 3′
JpncAs Reverse	5′ – GTGAACAACCCGACCCAG – 3′
pncA_LP3	5’ – GCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTC – ‘3
pncA_XP3	5’ – TTGCTCCACCGCCGCCAACAGTTCAT – ‘3

Results

The 34 fluorescent probes were successfully designed for the cover the entire *pncA* gene including upstream and downstream flanking regions (Table 2). These probes were be grouped into three groups according to their fluorophore labels (Quasar, Cal Red and Cal Orange) (Figure 1), Each ON probe was linked to an adjacent OFF probe (Figure 1). This design together with each probe having a specific Tm creates a distinct melt signature which is used to determine the presence or absence of a nucleotide change in the *pncA* gene and flanking regions.

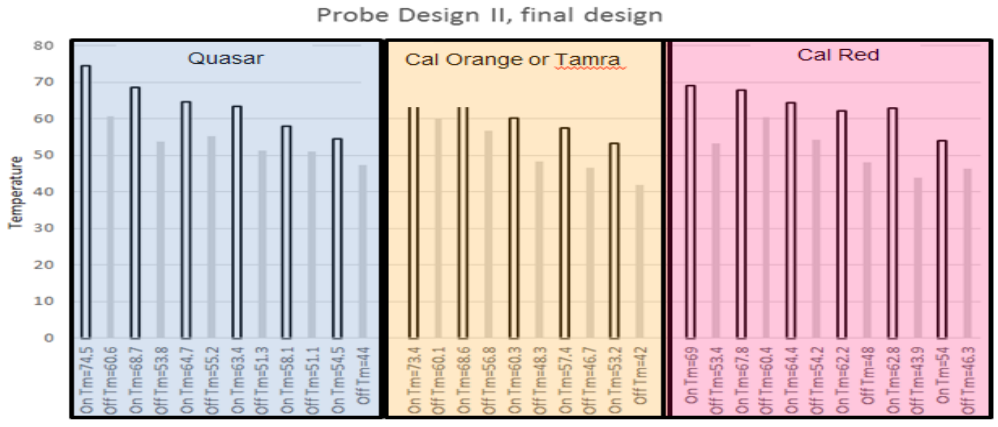

Figure 1. *pncA* probes in the three fluorescence colours showing each probes respective Tm.

Table 2. List of the *pncA* probes showing the fluorescence colour, Tm and probe sequence.

Fluorophore Colour	Probe name	Probe Tm (°C)	Probe Sequence
Quasar670	pncA_probe_196_215_ON	74,5	5′ – AACGCCGCGCTGGAGGAGATGCTT – 3′
Quasar670	pncA_probe_216_230_OFF	60,6	5′ – GGCCGATACCACCGT – 3′
Quasar670	pncA_probe_406_425_ON	68,7	5′ – ACCATCGGAGCGTACAGCGGCTGT – 3′
Quasar670	pncA_probe_426_440_OFF	53,8	5′ – CTACAAGGGTGCCTA – 3′
Quasar670	pncA_probe_161_180_ON	64,7	5′ – ACAGTTGGTTTGCAGCTCCTGAGT – 3′
Quasar670	pncA_probe_181_195_OFF	55,2	5′ – GCACTGCCGGCGTCG – 3′
Quasar670	pncA_probe_546_565_ON_bovis	63,4	5′ – AAGACCCGGGTGATCACTTCTCTT – 3′
Quasar670	pncA_probe_566_580_OFF	51,3	5′ – AAGGACTTCCACATC – 3′
Quasar670	pncA_probe_616_635_ON	58,1	5′ – AACGCTATCAGTGACTACCTGGTT – 3′
Quasar670	pncA_probe_636_650_OFF	51,1	5′ – CGCTGCGTTGGCTCG – 3′
Quasar670	pncA_probe_441_460_ON	54,5	5′ – AATCGGCAATTGAGTCGGTGTTTT – 3′
Quasar670	pncA_probe_461_475_OFF	47,3	5′ – CAGTCTGGACGCG – 3′
Cal Red610	pncA_probe_511_530_ON	69,0	5′ – ACTTCCTCGTCGTGGCCATCGCGT – 3′
Cal Red610	pncA_probe_531_345_OFF_bovis	53,4	5′ – CGGCACACTGGACTA – 3′
Cal Red610	pncA_probe_651_670_ON	67,8	5′ – AATCGCTGGCGGTAACTGGTGGTT – 3′
Cal Red610	pncA_probe_671_685_OFF	60,4	5′ – TTCTGCGAGGGTGGC – 3′
Cal Red610	pncA_probe_301_320_ON	64,4	5′ – AACATCGATCATTGTGTGCGCCTT – 3′
Cal Red610	pncA_probe_321_335_OFF	54,2	5′ – TGTGGTCGGTATTGC – 3′
Cal Red610	pncA_probe_336_355_ON	62,2	5′ – AGCGTGGCGTCGATGAGGTTGACT – 3′
Cal Red610	pncA_probe_356_370_OFF	48,0	5′ – AGTTGGCTGTGGCAA – 3′
Cal Red610	pncA_probe_231_250_ON	62,8	5′ – AAGACCTGACAGTGGGTGTGTCTT – 3′
Cal Red610	pncA_probe_251_265_OFF	43,9	5′ – ATCAGGGTGTTGGTG – 3′
Cal Red610	pncA_probe_721_740_ON	54,0	5′ – TTCCGAACTTATGGTGGACGTAAA – 3′
Cal Red610	pncA_probe_741_755_OFF	46,3	5′ – TTGCTTGGGCAGTTGC – 3′

Cal Orange560	pncA_probe_266_285_ON	73,4	5′ – TGGCGGTACGCAATGGCTTGGCCCA – 3′
Cal Orange560	pncA_probe_286_300_OFF	60,1	5′ – AGACGGCCGAGGAC – 3′
Cal Orange560	pncA_probe_581_600_ON	68,6	5′ – ACACCATCACGTCGTGGCAACCGT – 3′
Cal Orange560	pncA_probe_601_615_OFF	56,8	5′ – CCGAGGCGGCGGGCT – 3′
Cal Orange560	pncA_probe_476_495_ON	60,3	5′ – ACCTCTCGGCGTGGACTTCTATCCGT – 3′
Cal Orange560	pncA_probe_496_510_OFF	48,3	5′ – ATTGCGTTAGCGGTA – 3′
Cal Orange560	pncA_probe_686_705_ON	57,4	5′ – TTTCGTTGACGTGCAGAATGACAA – 3′
Cal Orange560	pncA_probe_706_720_OFF	46,7	5′ – TGCGGGTGTTGGTCA – 3′
Cal Orange560	pncA_probe_371_390_ON	53,2	5′ – AAAGAATGGTACGTCACTGCTGTT – 3′
Cal Orange560	pncA_probe_391_405_OFF	42,0	5′ – TCGGAGGAGTTGACG – 3′

To determine the diagnostic utility of the LATE-PCR assay, DNA from 414 phenotypically PZA susceptible and resistant *M. tuberculosis* isolates were amplified and sequenced. The Sanger sequencing method identified 184 (44.4%) isolates with a wild type *pncA* sequence and 230 (55.6%) isolates with a mutant *pncA* sequence. In total 76 different *pncA* mutations were identified, including insertions (n = 9), deletions (n = 5), single nucleotide polymorphisms (SNPs) (n = 54; 1 synonymous and 53 non-synonymous) as well as double SNPs (n = 8).

Table 3 shows the complete list detailing samples with their associated *pncA* genotype as well as whether a change in the LATE-PCR fluorescence signature was observed. Examples of fluorescence signatures for wild type and mutant isolates are shown in Figures 2-10.

Concordance between the LATE-PCR and Sanger sequencing results were observed in 406 (98.1%) isolates. LATE PCR and Sanger sequencing was repeated on the remaining 8 isolates. Two isolates which were originally found to be *pncA* wild type and had a change in the LATE-PCR melt curve, were upon repeating testing found to have a mutation in *pncA*. The remaining 3 isolates were found to have a shift in the LATE-PCR melt curve but were still wild type for *pncA* and 3 were found to not have a shift in the LATE-PCR melt curve but a nucleotide change in the *pncA* gene was again observed. This results in 179 of 182 wild-type isolates (98.4%) were correctly genotyped as *pncA* wild

type and 229 of 232 (98.7%) were correctly genotyped as *pncA* mutant. This implies a sensitivity of 98.7% (95% CI: 97.3 – 100) and specificity of 98.4% (95% CI: 96.5 – 100) assuming that the sequencing is correct and no heterogeneity was missed.

Discussion

The inclusion of PZA in the latest WHO MDR-TB treatment guidelines indicates that PZA will continue to play an important role in the treatment of drug susceptible (DS) and drug resistant (DR) TB. The lack of standardized routine PZA DST due to technical challenges means that a molecular-based diagnostic that investigates the *pncA* gene would provide the most insight into determining PZA susceptibility.

This study made use of non-symmetrical PCR method, which generates a large amount of single-stranded DNA of a specific target region. Fluorescent probes were designed to cover the single stranded DNA of the specific target region. These probes, which are separated by fluorescent colours and Tms, are melted off the single stranded DNA to produce a melt signature. The presence of a mutation in the specific target region should result in a shift in the Tm that the probe melts off and causes a shift in the melt signature.

This technique was successfully applied to the *pncA* gene and tested on a large cohort of purified DNA clinical isolates from South Africa. Examples of the generated melt signatures are given in the Figures 2 – 10. Each sample has signatures for the three different fluorescent colours namely; Quasar670; Cal Red610 and Cal Orange560. The changes in the melt signatures are indicated by black arrows. This method achieved excellent results, with a final sensitivity of 98.7% (95% CI: 97.3 – 100) and specificity of 98.4% (95% CI: 96.5 – 100). The test utility was not compromised by the position in the gene that was analysed. There was a good distribution of *pncA* SNPs across the entire *pncA* gene as shown in Figure 11, which is a good representation of the distribution of mutations observed globally (20).

Of the 6 isolates which showed discordant results, 3 were *pncA* wild type using Sanger sequencing but showed a shift in the LATE-PCR melt signature and 3 were *pncA* mutant using Sanger sequencing but showed no shift in the LATE-PCR melt signature. The 3 *pncA* wild type (as determined by Sanger

sequencing) isolates all had changes in the Cal Red fluorophore, however the melt signature shift may be due an underlying population that has a mutation in the *pncA* gene but we are unable to detect on the Sanger sequencing. This could be resolved using deep sequencing to identify this underlying population. The 3 *pncA* mutant isolates do not cluster together (codon 14, codon 152 and codon 187). In isolate R5526 the mutation at codon 187 (stop codon) was not detected because this mutation fall at the end of the probe thereby not influencing the Tm sufficiently to be detected. The other two mutations both have other isolates with mutations in the same codon which were identified. The may indicate that the probe has not effectively bound to the target strand of DNA and thus not resulted in a melt signature shift.

The excellent sensitivity and specificity coupled with a rapid turnaround time for a result (same day) means that LATE-PCR holds promise for a diagnostic to identify *pncA* mutations. LATE-PCR offers an attractive alternative to Sanger sequencing due to the rapid turnaround time in comparison to the Streicher *et al* manuscript (25). It also offers a much simpler alternative to whole genome sequencing (WGS) and next generation sequencing (NGS), as there is no need for expensive WGS/NGS sequencing equipment. However, we acknowledge some limitations. Firstly, the samples used in this study were purified DNA which requires culturing for 4-6 weeks as well as DNA extraction. Secondly, the full spectrum of mutations has not been evaluated. Thirdly, this technique needs to be validated using a more "raw" specimen type such as the Hain Lifescience Genolyse Kit for Genomic Bacterial DNA (Hain Lifescience, GmbH) from both sputum as well as culture.

Conclusion

We have successfully designed and tested a single closed-tube assay that is able to identify and distinguish the wild type *pncA* gene from strains with *pncA* genes containing genetic variants and thereby provides an accurate and rapid method to predict a PZA susceptible strain. Additional refinements will improve identification of the rare synonymous mutations and the few non-synonymous mutations in *pncA* that were not correctly scored as PZA resistance. This virtual sequencing method is a promising advance in novel diagnostics for PZA. However, many more *pncA* mutations need to be evaluated to confirm that the assay works optimally. We also need to further the

understanding of the mechanism of action to ensure that the assay is reliable enough to use in routine testing. This assay could be integrated alongside the GeneXpert or Line Probe Assay to determine the patient's resistance profile so that the treatment can be optimised.

Additional support: This study forms part of a bigger collaborative study, which includes an additional 240 *M. tuberculosis* strains from the United States of America (USA). The technique has been successfully replicated in multiple laboratories with similar successful as described in this study. The preliminary results of the isolates from the USA study show 217 of 240 (90.4%) samples correctly identified using the LATE-PCR with Lights-On/Lights-Off probes. There are 3 samples which are *pncA* wild type according to Sanger sequencing but called as having a *pncA* mutation, and there are 20 samples which are found to have *pncA* mutations according to Sanger sequencing but were called as *pncA* wild type. This results in a sensitivity of 98.2% (95% CI: 96.2 – 100) and a specificity of 72.6% (95% CI: 62.4 – 82.8).

Additional note: The assay could be modified so that silent or SNPs not associated with resistance can be masked using an additional probe which would bind preferentially when the mutant is present (out competes the mutant probe) which would result in a wild type melt signature.

Additional note: The correlation between Sanger sequencing and PZA DST should be noted. There was a total of 414 isolates. 50 isolates had no PZA DST data and were excluded for this calculation. The sensitivity was 98.4% (95% CI: 96.6 – 100) and specificity was 98.3% (95% CI: 96.4 – 100).

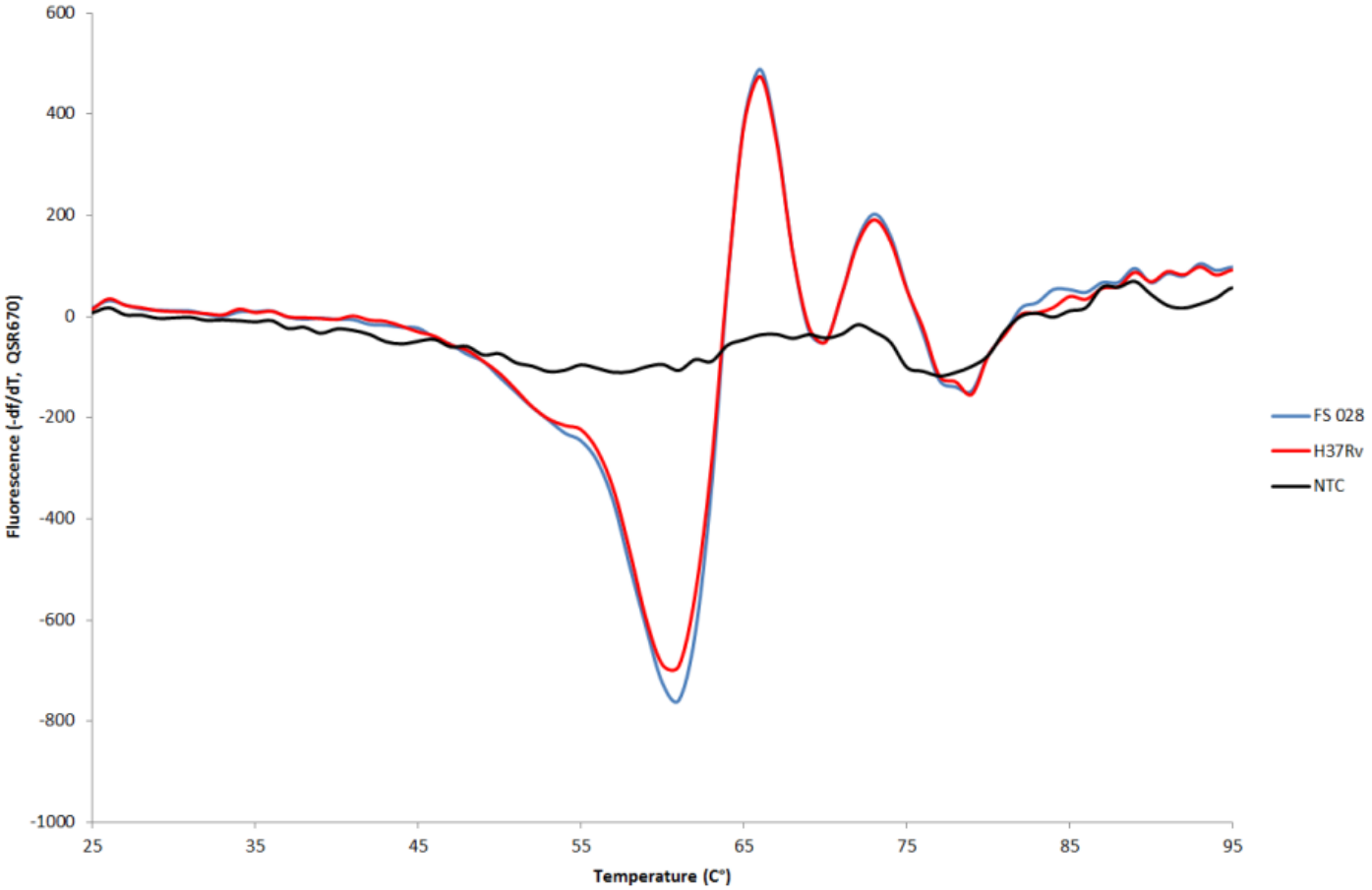

Figure 2. Quasar670 for sample FS 028 showing a wild type melt signature (Supp Table 1).

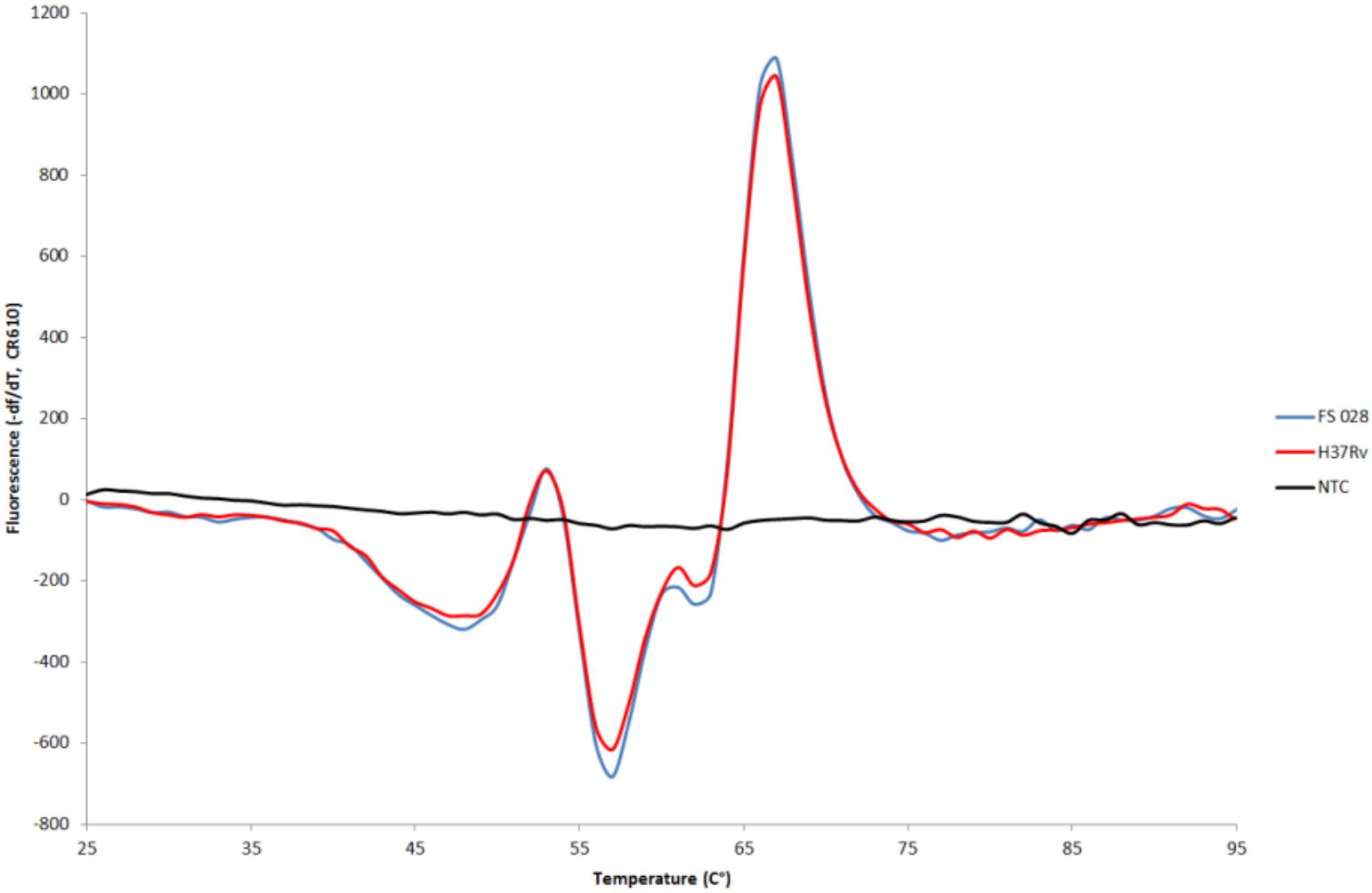

Figure 3. Cal Red610 for sample FS 028 showing a wild type melt signature (Supp Table 1).

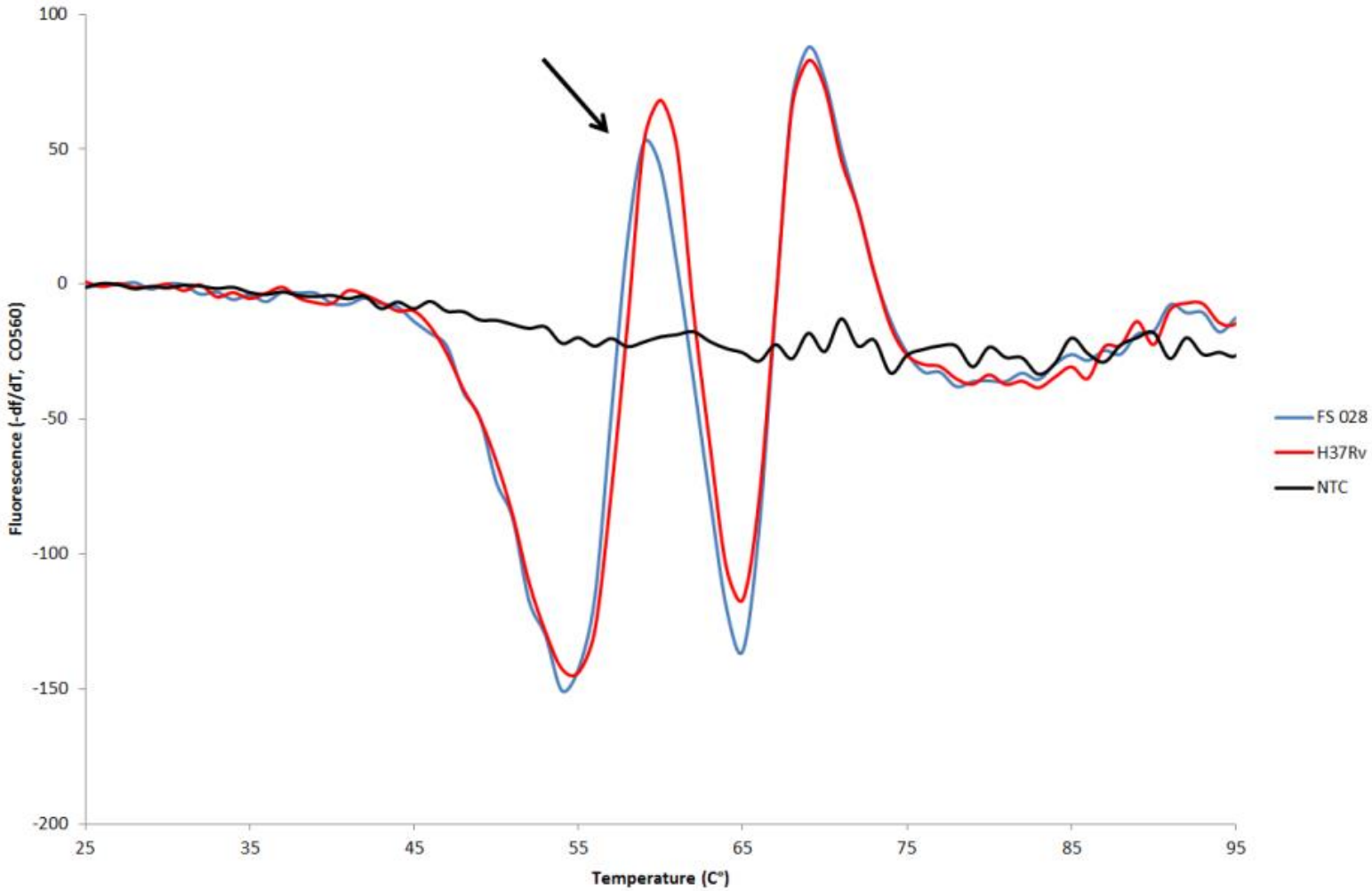

Figure 4. Cal Orange560 for sample FS 028 showing a shift in the melt signature, indicated by the black arrow (Supp Table 1).

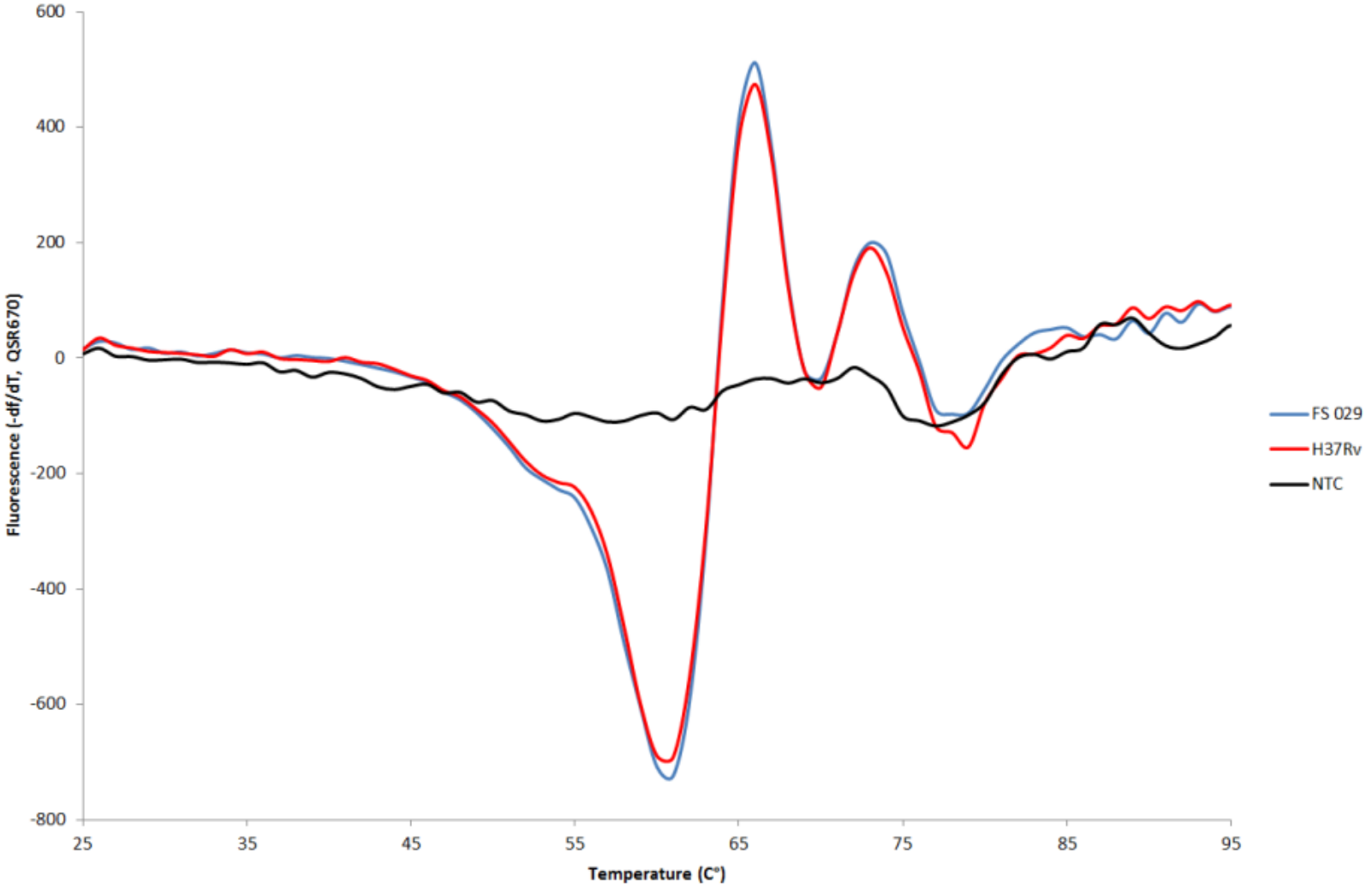

Figure 5. Quasar670 for sample FS 029 showing a wild type melt signature (Supp Table 1).

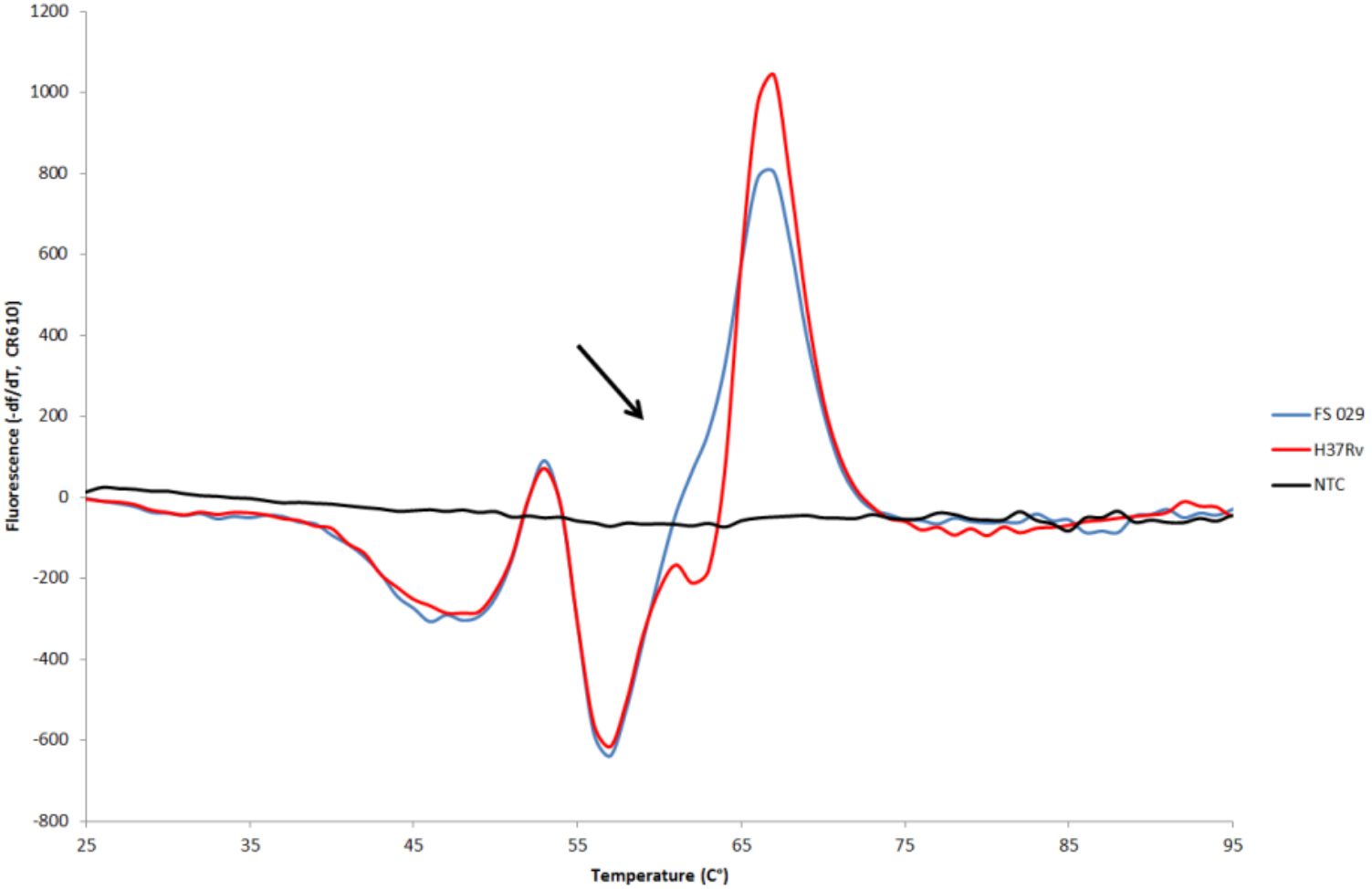

Figure 6. Cal Red610 for sample FS 029 showing a shift in the melt signature, indicated by the black arrow (Supp Table 1).

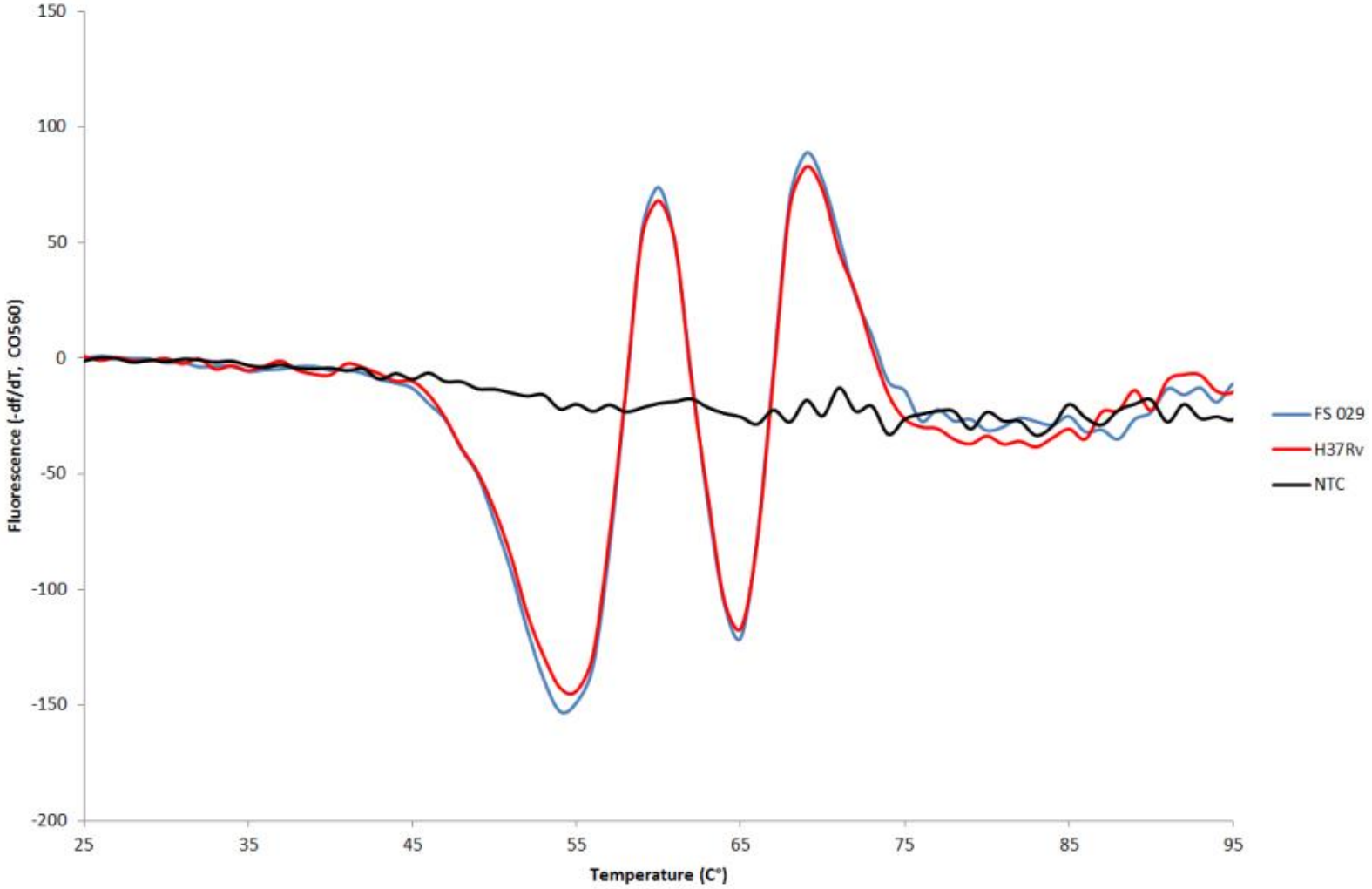

Figure 7. Cal Orange560 for sample FS 029 showing a wild type melt signature (Supp Table 1).

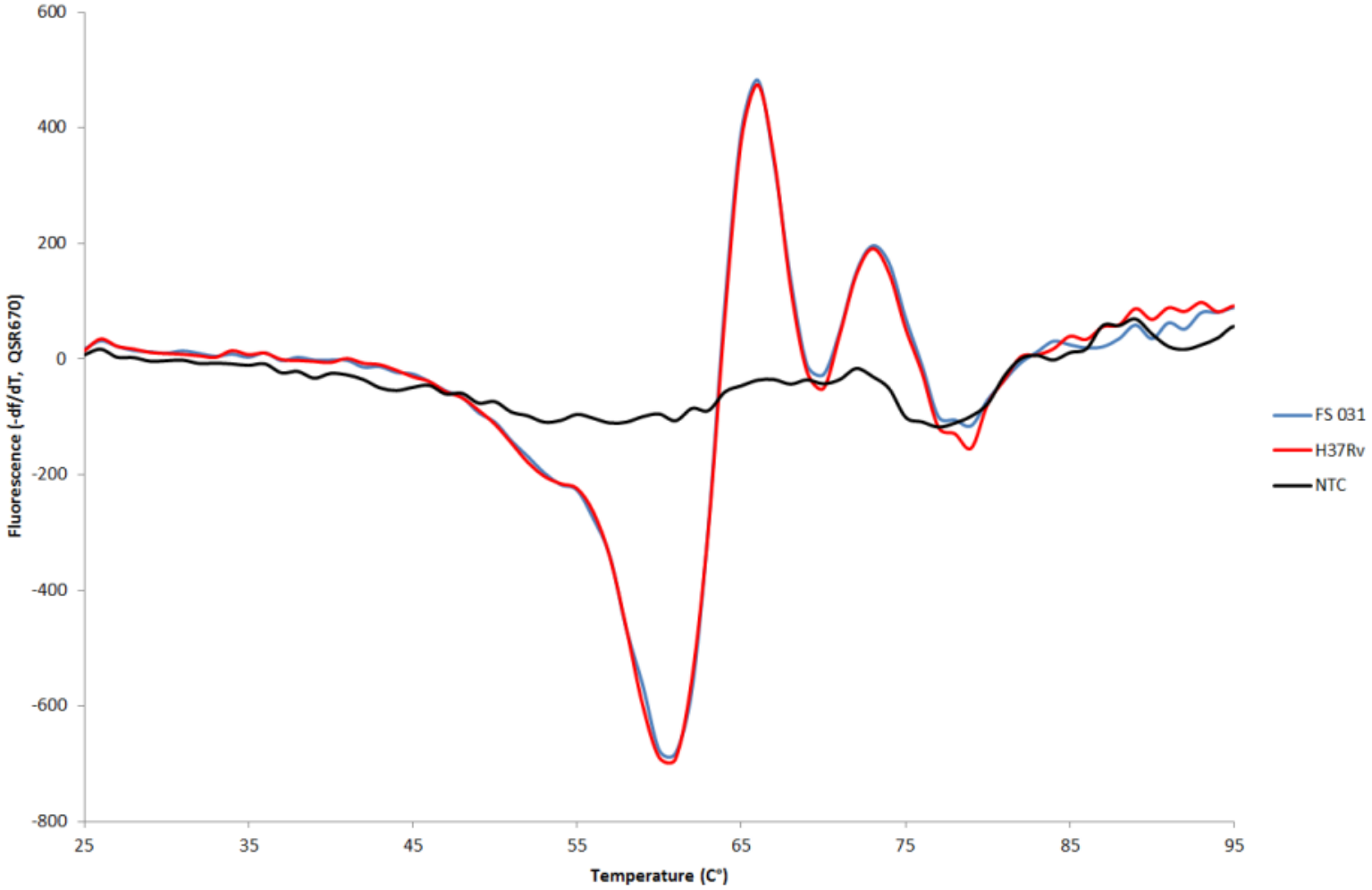

Figure 8. Quasar670 for sample FS 031 showing a wild type melt signature (Supp Table 1).

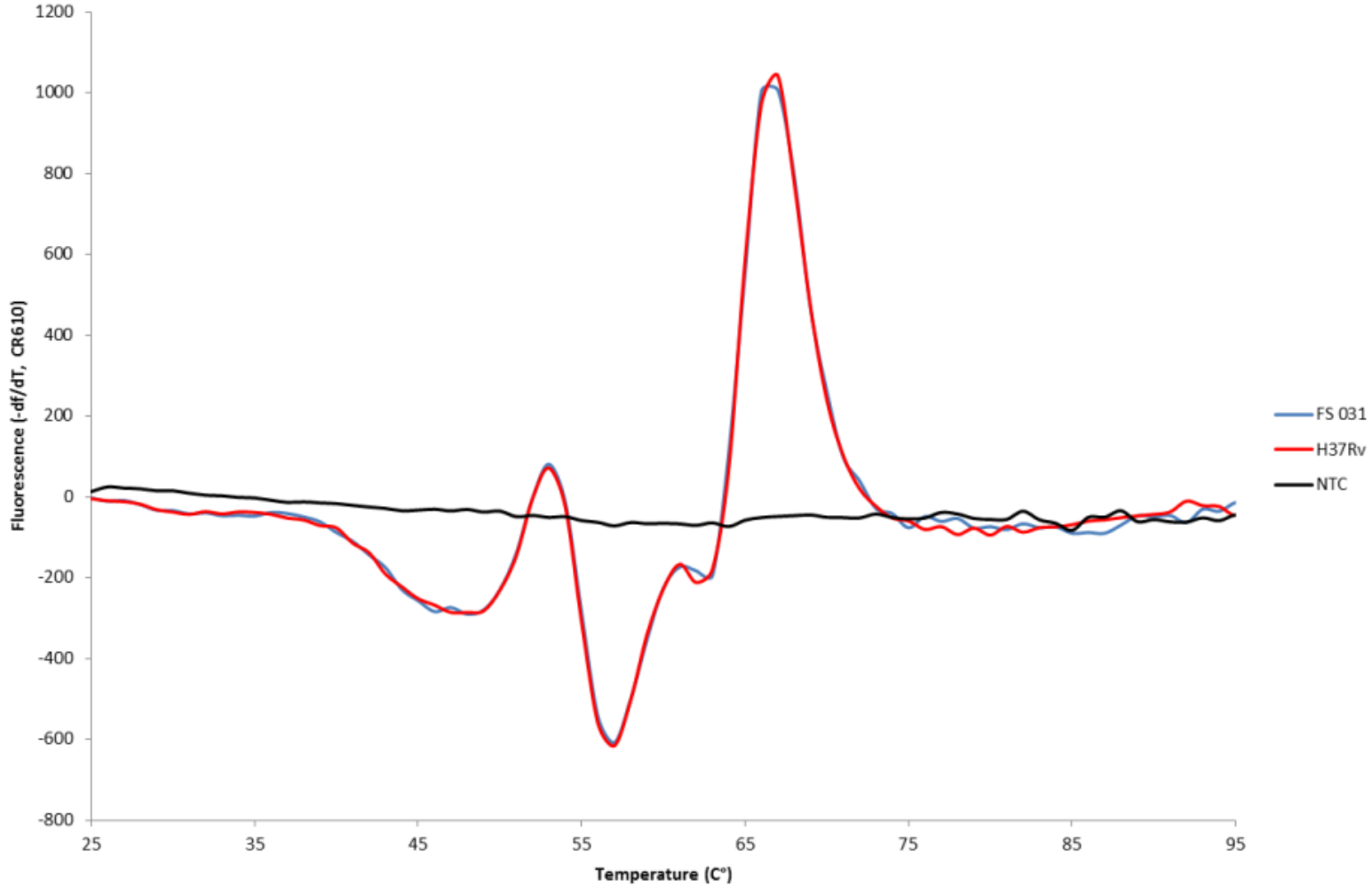

Figure 9. Cal Red610 for sample FS 031 showing a wild type melt signature (Supp Table 1).

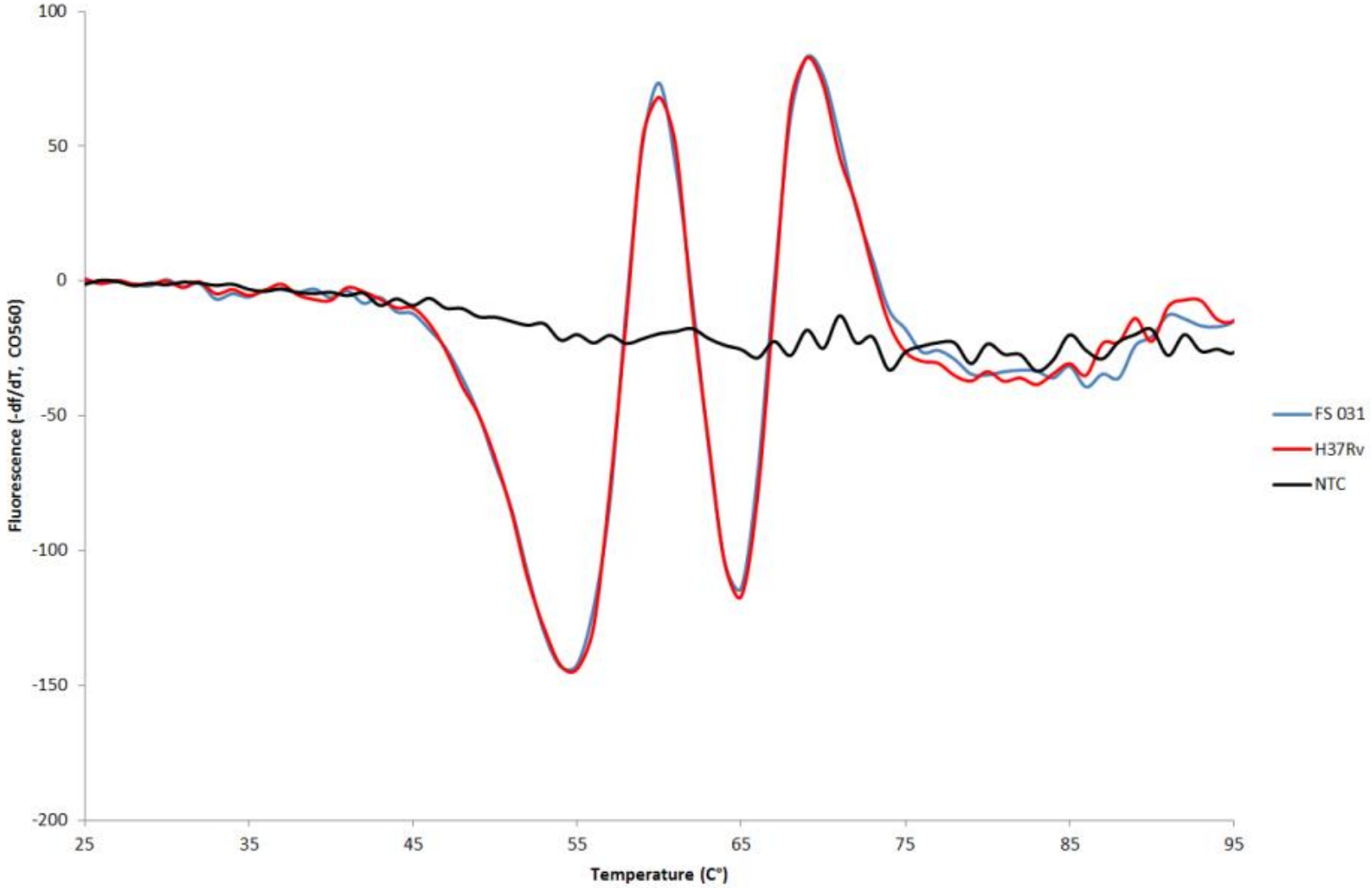

Figure 10. Cal Orange560 for sample FS 031 showing a wild type melt signature (Supp Table 1).

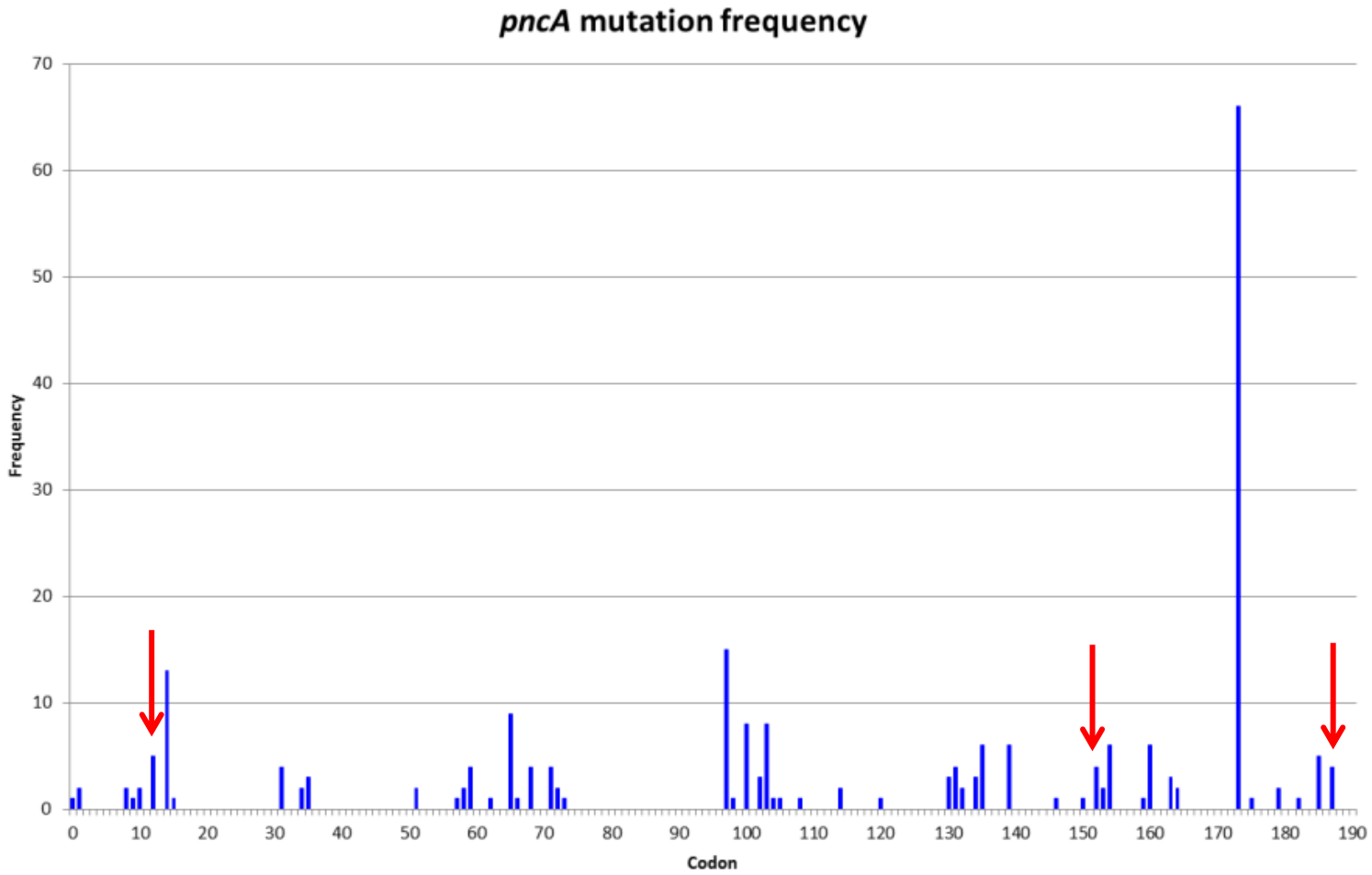

Figure 11. The distribution of tested *pncA* mutations and the frequency of each mutation. The three red arrows indicate the three codons which showed a discordant result.

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Supplemental Table 1. Sanger sequencing results of *pncA* gene with the changes observed with the Lights-On/Lights-Off probes.

Number	Sample	*pncA* mutation	Lights-On/Lights-Off change	Comment
1	EC 010A	INS: Codon 173, Position 1 (G)	QSR change	
2	EC 011.1	INS: Codon 173, Position 1 (G)	QSR change	
3	EC 011.2	INS: Codon 173, Position 1 (G)	QSR change	
4	EC 012A	Wild type	None	
5	EC 012B	Wild type	None	
6	EC 015	INS: Codon 173, Position 1 (G)	QSR change	
7	EC 016	INS: Codon 173, Position 1 (G)	QSR change	
8	EC 017	INS: Codon 173, Position 1 (G)	QSR change	
9	EC 018	Wild type	None	
10	EC 020	INS: Codon 173, Position 1 (G)	QSR change	
11	EC 021	INS: Codon 173, Position 1 (G)	QSR change	
12	EC 022	Gly102Val	QSR change	
13	EC 023	INS: Codon 173, Position 1 (G)	QSR change	
14	EC 024	Wild type	None	
15	EC 025	INS: Codon 173, Position 1 (G)	QSR change	
16	EC 026	INS: Codon 173, Position 1 (G)	QSR change	
17	EC 029	Wild type	None	
18	EC 031	Thr100Pro and STOP187Trp	QSR change	
19	EC 031A	Cys14Arg	CR, CO change	
20	EC 035	Leu120Pro	CR change	
21	EC 039	INS: Codon 173, Position 1 (G)	QSR change	
22	EC 040	Ile131Ser	CO change	
23	EC 041	Thr100Pro and STOP187Trp	CR change	
24	EC 042	INS: Codon 173, Position 1 (G)	QSR change	
25	EC 043	Cys14Arg	QSR, CR, CO change	
26	EC 045	Wild type	None	
27	EC 046	INS: Codon 173, Position 1 (G)	QSR change	
28	EC 048	Wild type	None	
29	EC 048A	Wild type	None	
30	EC 049	INS: Codon 173, Position 1 (G)	QSR change	
31	EC 050	Cys14Arg	CR change	
32	EC 051	Thr135Pro	CR change	
33	EC 051B	Thr135Pro	CR change	

34	EC 052	INS: Codon 173, Position 1 (G)	QSR change	
35	EC 053	Wild type	None	
36	EC 054	Wild type	None	
37	EC 055	Wild type	None	
38	EC 056	INS: Codon 173, Position 1 (G)	QSR change	
39	EC 057	INS: Codon 173, Position 1 (G)	QSR change	
40	EC 058	Cys14Arg	QSR change	
41	EC 059	Thr100Pro and STOP187Trp	CR change	
42	EC 061	Tyr103His	QSR change	
43	EC 062	INS: Codon 173, Position 1 (G)	QSR change	
44	EC 063	INS: Codon 173, Position 1 (G)	QSR change	
45	EC 065	Wild type	None	
46	EC 066	Wild type	None	
47	EC 067	Thr135Pro	CR change	
48	EC 068	Wild type	None	
49	EC 069	INS: Codon 173, Position 1 (G)	QSR change	
50	EC 070	INS: Codon 173, Position 1 (G)	QSR change	
51	EC 071	Wild type	None	
52	EC 072	INS: Codon 173, Position 1 (G)	QSR change	
53	EC 072B	INS: Codon 173, Position 1 (G)	QSR change	
54	EC 073	Wild type	None	
55	EC 074	INS: Codon 173, Position 1 (G)	QSR change	
56	EC 075	INS: Codon 173, Position 1 (G)	QSR change	
57	EC 076	Gly108Arg	CO change	
58	EC 077	INS: Codon 173, Position 1 (G)	QSR change	
59	EC 080	Wild type	None	
60	EC 081	INS: Codon 173, Position 1 (G)	QSR change	
61	EC081A	INS: Codon 173, Position 1 (G)	QSR change	
62	EC 082	Wild type	None	
63	EC 083	Wild type	None	
64	EC 084	INS: Codon 173, Position 1 (G)	QSR change	
65	EC 085	Wild type	None	
66	EC 086	Ser65Ser	CR change	
67	EC 087	Wild type	CR change	
68	EC 088	Wild type	None	
69	EC 090	Wild type	None	

70	EC 091	INS: Codon 173, Position 1 (G)	QSR change	
71	EC 092	Cys72Gly	CR change	
72	EC 093	INS: Codon 173, Position 1 (G)	QSR change	
73	EC 095	Wild type	None	
74	EC 096	INS: Codon 173, Position 1 (G)	QSR change	
75	EC 097	INS: Codon 173, Position 1 (G)	QSR change	
76	EC 098	Cys14Arg	CR, CO change	
77	EC 098A	Cys14Arg	CR, CO change	
78	EC 099	INS: Codon 173, Position 1 (G)	QSR change	
79	EC 100	INS: Codon 173, Position 1 (G)	QSR change	
80	EC 104	Wild type	None	
81	EC 105	INS: Codon 173, Position 1 (G)	QSR change	
82	EC 106	Wild type	None	
83	EC 108	INS: Codon 173, Position 1 (G)	QSR change	
84	EC 109	INS: Codon 173, Position 1 (G)	QSR change	
85	EC 110	INS: Codon 173, Position 1 (G)	QSR change	
86	EC 111	Glu10Pro	CR, CO change	
87	EC 113A	Leu182Ser	CR change	
88	EC 114	Thr114Met	QSR, CR, CO change	
89	EC 115	INS: Codon 173, Position 1 (G)	QSR, CR change	
90	EC 116	Glyc97Asp	QSR, CO change	
91	EC 303	Cys14Arg	CR, CO change	
92	EC 304	Wild type	None	
93	EC 305	Ala102Val	QSR change	
94	EC 307A	Wild type	None	
95	EC 310	INS: Codon 173, Position 1 (G)	QSR, CR change	
96	EC 311	Trp68Arg	CR change	
97	EC 312	Wild type	None	
98	EC 317	Val139Gly	CO change	
99	EC 338B	INS: Codon 173, Position 1 (G)	QSR change	
100	EC 342	Wild type	None	
101	EC 347	Wild type	None	
102	EC 358	INS: Codon 173, Position 1 (G)	QSR change	
103	EC 364	Wild type	None	
104	EC 365	Tyr103His	QSR, CO change	
105	EC 367	Ala98Val and Met175Val	QSR, CO change	

106	EC 368B	INS: Codon 173, Position 1 (G)	QSR, CO change	
107	EC 372	Leu35Arg	CO change	
108	EC 373	Wild type	None	
109	EC 378	Leu35Arg	CO change	
110	EC 379	INS: Codon 173, Position 1 (G)	QSR change	
111	EC 379B	INS: Codon 173, Position 1 (G)	QSR change	
112	EC 382	Wild type	None	
113	EC 382A	Wild type	None	
114	EC 383A	Thr100Pro and STOP187Val	CR change	
115	EC 385A	INS: Codon 173, Position 1 (G)	QSR change	
116	EC 385B	INS: Codon 173, Position 1 (G)	QSR change	
117	EC 386B	INS: Codon 173, Position 1 (G)	QSR change	
118	EC 387	Wild type	None	
119	EC 503	Wild type	None	
120	EC 504	Ser59Pro	QSR, CR change	
121	EC 514	Ser104Arg	CR change	
122	EC 525	Wild type	None	
123	EC 525A	Wild type	None	
124	EC 531B	Wild type	None	
125	EC 536B	Pro62Leu	CO change	
126	EC 541A	INS: Codon 173, Position 1 (G)	QSR, CO change	
127	EC 548B	Wild type	None	
128	EC 549	INS: Codon 173, Position 1 (G)	QSR, CO change	
129	EC 550	INS: Codon 173, Position 1 (G)	QSR change	
130	EC 550A	INS: Codon 173, Position 1 (G)	QSR change	
131	EC 550B	INS: Codon 173, Position 1 (G)	QSR change	
132	EC 557	INS: Codon 173, Position 1 (G)	QSR change	
133	EC 559	Wild type	None	
134	EC 561	Ser65Ser	CR change	
135	EC 565	Wild type	None	
136	EC 565A	Wild type	None	
137	EC 567A	Wild type	None	
138	EC 569A	Wild type	None	
139	EC 569B	Wild type	None	
140	EC 573B	INS: Codon 173, Position 1 (G)	QSR change	
141	EC 577	Wild type	None	

142	EC 578	INS: Codon 173, Position 1 (G)	CR change	
143	EC 580	INS: Codon 173, Position 1 (G)	QSR change	
144	EC 583	Ile31Ser	CO change	
145	EC 589	Wild type	None	
146	EC 589A	Cys14Arg	CR change	
147	EC 594A	INS: Codon 173, Position 1 (G)	QSR change	
148	EC 610	Wild type	None	
149	FS 001	Thr135Pro	CR change	
150	FS 002	Gly97Cys	QSR change	
151	FS 003	Wild type	None	
152	FS 004	Ser59Pro	CR change	
153	FS 006	Arg154Gly	CR change	
154	FS 007	Wild type	None	
155	FS 007:2	Wild type	CR change	Discordant
156	FS 008	Wild type	None	
157	FS 009	Wild type	None	
158	FS 010	Trp68Gly	CR change	
159	FS 010:2	Trp68Gly	CR change	
160	FS 011	Wild type	None	
161	FS 011.2	Wild type	None	
162	FS 012	Thr100Ile and Thr160Ala	QSR + CR change	
163	FS 012:2	Thr100Ile and Thr160Ala	QSR, CR change	
164	FS 012:5	Thr100Ile and Thr160Ala	QSR, CR change	
165	FS 014	Thr100Ile and Thr160Ala	QSR + CR change	
166	FS 015	Tyr103STOP	QSR change	
167	FS 016	INS: Codon 12, Position 2 (G)	CO change	
168	FS 017	Wild type	None	
169	FS 018	INS: Codon 173, Position 1 (G)	CR change	
170	FS 019	Wild type	None	
171	FS 020	Wild type	None	
172	FS 021	Ala134Val	CR change	
173	FS 021A	Ala134Val	CR change	
174	FS 022	INS: Codon 131, Position 2 (GT)	CR change	
175	FS 023	INS: Codon 12, Position 2 (G)	QSR, CR, CO change	
176	FS 024	Wild type	None	
177	FS 025	Wild type	None	

178	FS 026	Wild type	None	
179	FS 027	Wild type	None	
180	FS 028	INS: Codon 12, Position 2 (G)	CO change	Fig 2-4
181	FS 029	Val139Ala	CR change	Fig 5-7
182	FS 031	Wild type	None	Fig 8-10
183	FS 031:2	Asn118Ser	QSR, CR, CO change	
184	FS 032	Wild type	None	
185	FS 033	Wild type	None	
186	FS 034	Ile31Asn	CO change	
187	FS 035	Wild type	None	
188	FS 036	Wild type	None	
189	FS 036A	Tyr103STOP	QSR change	
190	FS 037	His51Pro	CR change	
191	FS 038	Wild type	CR change	Discordant
192	FS 038A	Wild type	None	
193	FS 039	Wild type	None	
194	FS 040	Wild type	None	
195	FS 041	Wild type	None	
196	FS 042	Ser65Ser	CR change	
197	FS 043	Wild type	None	
198	FS 043A	Wild type	None	
199	FS 044	Gly132Ser	CR change	
200	FS 045	Wild type	None	
201	FS 046	INS: Codon 131, Position 1 (G)	CR change	
202	FS 047	Wild type	None	
203	FS 048	Wild type	None	
204	FS 049A	Wild type	None	
205	FS 050	Wild type	None	
206	FS 051	Ser65Ser	CR change	
207	FS 052	Promotor region: -11, A>G	CR change	
208	FS 053	Wild type	None	
209	FS 054	Wild type	None	
210	FS 055	Wild type	None	
211	FS 056	Arg154Gly	CR change	
212	FS 059	Wild type	None	
213	FS 060	Wild type	None	

214	FS 061	Wild type	None	
215	FS 062	DEL: Codon 153, Position 3 (8bp)	CR change	
216	FS 063	Glyc97Asp	QSR change	
217	FS 064	Wild type	None	
218	FS 065	Ser59Pro	CR change	
219	FS 067	Wild type	None	
220	FS 068	Wild type	None	
221	FS 301	Wild type	None	
222	FS 303	Wild type	None	
223	FS 304	Leu35Arg	CO change	
224	FS 305	DEL: Codon 130, Position 2 (T)	QSR, CR change	
225	FS 306	Wild type	None	
226	FS 308	Wild type	None	
227	FS 309	Cys14Arg	None	Discordant
228	FS 310	Wild type	None	
229	FS 311	DEL: Codon 163, Position 3 (11bp)	CR change	
230	FS 314	Wild type	None	
231	FS 315	Wild type	None	
232	FS 321	Wild type	None	
233	FS 322	Wild type	None	
234	FS 331	DEL: Codon 163, Position 3 (11bp)	CR change	
235	FS 331B	DEL: Codon 163, Position 3 (11bp)	CR change	
236	FS 336	Promoter, -7: T>C	CR change	
237	FS 338	Glyc97Asp	QSR change	
238	FS 348	Thr135Pro	CR change	
239	FS 351	Glyc97Asp	QSR change	
240	FS 352	Wild type	None	
241	FS 353	Wild type	None	
242	FS 355	Wild type	None	
243	FS 356	Thr135Pro	CR change	
244	FS 358	INS: Codon 12, Position 2 (G)	CO change	
245	FS 359	Ser65Ser	CR change	
246	FS 500	Phe58Ser	CR, CO change	
247	FS 501	Wild type	None	
248	FS 503	Ser59Pro	CR change	
249	FS 507	Wild type	None	

250	FS 508	Wild type	None	
251	FS 512	Wild type	None	
252	FS 513	Arg154Gly	CR change	
253	FS 519	Glyc97Asp	QSR change	
254	FS 527	Wild type	None	
255	FS 535	Wild type	None	
256	FS 537	Wild type	None	
257	FS 550	Wild type	None	
258	FS 551	Wild type	None	
259	FS 564	Wild type	None	
260	FS 579	Wild type	None	
261	FS 581	His51Pro	CR change	
262	FS 582	Wild type	None	
263	FS 583	Wild type	None	
264	FS 583A	Wild type	None	
265	FS 584	Arg154Gly	CR change	
266	FS 585	Glyc97Asp	QSR change	
267	FS 588	Val73Ile	CR change	
268	FS 588A	Wild type	None	
269	FS 591	Arg154Gly	CR change	
270	FS 592	Wild type	None	
271	FS 595A	INS: Codon 173, Position 1 (G)	QSR change	
272	FS 596	Wild type	None	
273	FS 598	Wild type	None	
274	FS 598A	Wild type	None	
275	GA 001	Wild type	None	
276	GA 002	Wild type	None	
277	GA 003	Wild type	None	
278	GA 004	Wild type	None	
279	GA 006A	Wild type	None	
280	GA 007	Wild type	None	
281	GA 008	Arg154Gly	QSR, CR change	
282	GA 009	INS: Codon 152, Position 3 (C)	None	Discordant
283	GA 010	Glyc97Asp	QSR, CO change	
284	GA 012	Wild type	None	
285	GA 013A	Wild type	None	

286	GA 016	Wild type	None	
287	GA 017	Val139Gly	CR change	
288	GA 018	Val139Gly	CR, CO change	
289	GA 020	Thr100Pro	QSR change	
290	GA 022	Wild type	None	
291	GA 024	Wild type	None	
292	GA 027	Wild type	None	
293	GA 029	INS: Codon 152, Position 3 (C)	CR, CO change	
294	GA 029A	INS: Codon 152, Position 3 (C)	CO change	
295	GA 030	His71Tyr	CR change	
296	GA 032	Wild type	None	
297	GA 035	His71Tyr	CR change	
298	GA 036	Wild type	None	
299	GA 037	Wild type	None	
300	GA 040	Wild type	None	
301	GA 041	Glyc97Asp	QSR change	
302	GA 042	Wild type	None	
303	GA 043	Wild type	None	
304	GA 044	Wild type	None	
305	GA 045	INS: Codon 173, Position 1 (G)	QSR, CR change	
306	GA 046	Wild type	None	
307	GA 046A	Wild type	None	
308	GA 047	His71Tyr	CR change	
309	GA 048	Wild type	None	
310	GA 050	INS: Codon 173, Position 1 (G)	QSR change	
311	GA 051	His71Tyr	CR change	
312	GA 056	Wild type	CR change	Discordant
313	GA 057	Wild type	None	
314	GA 058	Wild type	None	
315	GA 059	Wild type	None	
316	GA 060	Wild type	None	
317	GA 061	Wild type	None	
318	GA 062	Wild type	None	
319	GA 063	INS: Codon 152, Position 3 (C)	CO change	
320	GA 064	Glyc97Cys	QSR change	
321	GA 064A	Gly97Cys	QSR change	

322	GA 065	Wild type	None	
323	GA 068	Wild type	None	
324	GA 069	Wild type	None	
325	GA 074	Cys14Arg	CR, CO change	
326	GA 076	Wild type	None	
327	GA 078	Wild type	None	
328	GA 080	Wild type	None	
329	GA 302	Ala134Asp	QSR change	
330	GA 306	Wild type	None	
331	GA 315	Wild type	None	
332	GA 316	Wild type	None	
333	GA 321	Wild type	None	
334	GA 322	Tyr34Asp	QSR, CR change	
335	GA 324	Ala102Val	CR change	
336	GA 329	Wild type	None	
337	GA 331	Cys14Arg	CO change	
338	GA 342	Wild type	None	
339	GA 346	Wild type	None	
340	GA 359	DEL: Codon 130, Position 2 (T)	CR change	
341	GA 360	Glyc97Asp	QSR change	
342	GA 369	Wild type	None	
343	GA 500	Wild type	None	
344	GA 502	Wild type	None	
345	GA 504	Wild type	None	
346	GA 509	Wild type	None	
347	GA 514	Wild type	None	
348	GA 516	Wild type	None	
349	GA 517	INS: Codon 173, Position 1 (G)	QSR change	
350	GA 523	Wild type	None	
351	GA 525	Ser66Pro	CR, CO change	
352	GA 527A	Ser65Ser	CR change	
353	GA 531	Wild type	None	
354	GA 532	Wild type	None	
355	GA 535	Wild type	None	
356	GA 536	INS: Codon 12, Position 2 (G)	CO change	
357	GA 537	Wild type	None	

358	GA 540	Wild type	None	
359	GA 540A	Wild type	None	
360	GA 567A	Ser65Ser	QSR, CR change	
361	GA 567B	Wild type	None	
362	GA 569	Wild type	None	
363	1093	Thr114Met	CO change	
364	R 1	INS: Codon 131, Position 1 (GG)	CR change	
365	R 32	Leu159Pro and Ser185Thr	CR change	
366	R 40	INS: Codon 130, Position 1 (G)	CR change	
367	R 44	Trp68Gly and Ser185Thr	CR change	
368	R 65	Ser65Ser	CR change	
369	R 77	Ser65Ser	CR change	
370	R 103	Ser185Thr	CR change	
371	R 166	DEL: Codon 58, Position 1 (T) and Ser179Arg	QSR, CR change	
372	R 201	Wild type	None	
373	R 224	Cys72Tyr	QSR change	
374	R 238	Wild type	None	
375	R 296	Wild type	None	
376	R 309	DEL: Codon 58, Position 1 (T)	QSR change	
377	R 350	Ser179Arg	QSR change	
378	R 357	Ser185Thr	CR change	
379	R 374	Val9Leu	CR change	
380	R 394	Cys14Arg	CR change	
381	R 455	His57Tyr and Ser185Thr	CR change	
382	R 484	Tyr103His	QSR change	
383	R 510	Tyr103STOP	QSR change	
384	R 10 247	Wild type	None	
385	R 1 142	DEL: Codon 153, Position 3 (8bp)	CR change	
386	R 15 487	Wild type	None	
387	R 2 654	Ala146Thr	CO change	
388	R 2 757	Tyr103His	CR change	
389	R 3 384	Ser164Pro	QSR change	
390	R 3 989	Met1Thr	CO change	
391	R 5 065	Gly132Ala	CR change	
392	R 5 526	STOP187Tyr	None	Discordant
393	R 9 339	Wild type	None	

394	TT 8	Gly150Gly	CR change	
395	TT 39	Tyr34STOP	CR change	
396	TT 227	INS: Codon 152, Position 3 (G)	QSR change	
397	TT 233	IIe31Ser	CR change	
398	X 2	Cys14Arg	CR change	
399	X 6	DEL: Codon 8, Position 1 (G)	CO change	
400	X 9	INS: Codon 131, Position 1 (GG)	CR change	
401	X 10	Thr100Ile and Thr160Ala	QSR, CR change	
402	X 16	Val139Met	CR change	
403	X 17	INS: Codon 97, Position 1 (GG)	QSR change	
404	X 23	Val139Met	CR change	
405	X 24	Gly105Asp	QSR, CR change	
406	X 52	Tyr103His	QSR change	
407	X 70	Gly97Asp	QSR change	
408	X 74	Asp8Asn	CO change	
409	X130	Thr100Ile and Thr160Ala	QSR change	
410	X 136	Gly97Asp	CO change	
411	X 138	Gln10Pro	CO change	
412	X 160	Met1Thr	CO change	
413	X 167	Ser164Pro	QSR change	
414	X 199	INS: Codon 173, Position 1 (G)	QSR change	

Abbreviations: QSR, Quasar; CR, Cal Red; CO, Cal Orange.

Chapter 7

General Conclusion

General Discussion

Pyrazinamide (PZA) continues to form a vital component of the anti-TB treatment regimen. However, there is a significant lack of data regarding the actual incidence of PZA resistance, as noted in the WHO global report. The prevalence of PZA resistance by region as well as globally was largely unknown. This was due to the lack of reliable, standardized routine PZA DST. To address this knowledge gap we performed a systematic literature review and thereby estimated the rates of PZA resistance for each WHO region (Africa, Americas, Eastern Mediterranean, Europe, South East Asia, Western Pacific) as well as the pooled estimate for any TB; high-risk of MDR-TB and lastly MDR-TB (1). Our findings showed a clear increase of PZA resistance in the groups (any TB; high-risk MDR-TB and MDR-TB) from 16.2% to 41.3% to 60.5% was observed. This was the first study to show the global estimates, and thereby presented novel and necessary data to inform on the prevalence of PZA resistance. The summary estimates of the MDR-TB group from the systematic review were found to be similar to those observed in a previous systematic review where they estimate PZA resistance prevalence to be at 51% (range 31% - 89%) (2). More recently a study observed an association between PZA resistance and MDR-TB in a multi-country surveillance project with comparable rates of PZA resistance (3).

The importance of PZA in treatment has been recently reiterated by the WHO's recommendation of a shortened MDR-TB regimen as well as clinical trials evaluating the efficacy of new regimens (4-6). This highlights the need for routine standardized PZA DST particularly in patients with MDR-TB where the strong association was observed with PZA resistance (1, 2). This is crucial when considering possible novel drug combinations (PaMZ) for the treatment of MDR-TB. Our systematic review (Chapter 2) highlighted the complexity of PZA resistance on a global scale in terms of genotypic identification of PZA resistance. To date more than 600 different mutations occurring at 397 distinct positions in the *pncA* gene have been described (1, 7). The fact that there are so many different mutations means that understanding how each mutation is related to microbiological resistance is complex and still largely unknown. Furthermore, the clinical relevance of microbiological PZA resistance remains unknown with the exception of that patients with isolates with a raised PZA MIC have a poorer outcome.

In chapter 3 we aimed to determine the prevalence of PZA resistance in a cohort of South African patients with a spectrum of different resistance phenotypes (pan-susceptible to XDR-TB). A significant increase of prevalence of PZA resistance from 0% (pan-susceptible) to 96% (XDR-TB) was shown. This clearly shows that the inclusion of PZA in the treatment regimen of most MDR- and XDR-TB cases is unlikely to provide therapeutic benefit. Our study makes a very strong case for routine PZA DST particularly in rifampicin resistant cases to confirm whether the inclusion of PZA in the treatment should be counted. Recently, targeted gene sequencing of the *pncA* gene has been suggested to be a rapid alternative to the technically challenging culture based DST method (8). In order to address this, our study investigated the correlation between phenotypic and genotypic data. The overall sensitivity was 95.0% (95% CI 92.1–98.0) and specificity was 99.1% (95% CI 98.4–99.9), indicating that targeted *pncA* sequencing could be used to identify PZA resistance (9). The rates of predicting PZA resistance using *pncA* Sanger sequencing as described above relate well to previous studies, such as a recent study where they could predict between 85-90% of PZA resistance when using *pncA*. This is particularly significant as the study investigated around 5000 patients across 5 different countries. This rate has been shown in previous studies across the globe (10-17). While no obvious association between strain lineage and PZA susceptibility. However the link between a strain lineage and a *pncA* mutation may be indicative of transmission.

We propose that targeted *pncA* sequencing could be readily implemented into the routine diagnostic laboratory with a turnaround time of less than 48 hours from smear positive specimens (8). However, smear negative specimens would require prior MGIT culture which is in line with the current diagnostic guidelines for confirmation of rifampicin resistance using the MTBDR*plus* line probe assay.

A significant knowledge gap existed in the literature as to which of the *pncA* mutations did not confer resistance at the standardized PZA concentration of 100 μg/ml. This largely resulted from the poor reliability of the PZA DST. Chapter 4 describes 10 SNPs which were identified that were not involved in conferring resistance to PZA at 100 μg/ml. These 10 SNPs include both non-synonymous and

synonymous SNPs. However, this list may be larger than the 10 SNPs identified in this study and to address this, we collated a comprehensive list of all possible *pncA* mutations which have been reported not to confer resistance (18). Together this further highlights the need to decipher the role of each mutation in *pncA* in PZA resistance such that genetic tests can be accurately interpreted.

Chapter 5 investigates the fitness cost of SNPs and large deletions located in the *pncA* gene. A significant difference in growth rate was observed. This crude measure of growth rate suggests a possible fitness cost to the organism. This fitness cost may translate to the diminished ability to transmit. However, preliminary data from the *pncA* large deletion isolates suggests that these changes in the gene do not prevent transmission. Understanding the impact of compensatory mutations associated with PZA resistance will be crucial for understanding how PZA resistant strains are transmitted. This differs from previous studies which have suggested that in vitro growth rate is a surrogate for fitness cost and the inability to transmit (19).

Lastly, in Chapter 6 we developed (in collaboration with Brandeis University) and evaluated a novel rapid PZA diagnostic, using a combination of LATE-PCR technology and lights-on/lights-off probes. Fluorescent probes were successfully designed to cover the *pncA* gene and this assay was assessed covering over 50 different *pncA* mutations described using Sanger sequencing. The study produced an overall sensitivity of 98.7% (95% CI: 97.3 – 100) and specificity of 98.4% (95% CI: 96.5 – 100). This compares favourably with targeted *pncA* sequencing (8). We conclude that this technology could be used to rapidly identify patients with TB that are susceptible to PZA. However, we have noted certain limitations. In this study we used purified DNA which excludes the presence of possible PCR inhibitors and contaminates that could influence the fluorescent signatures. Our method now needs to be validated with DNA extracted from clinical specimens.

Overall this thesis presents together new knowledge of PZA resistance in *M. tuberculosis*. Several key knowledge gaps have been addressed, however many more questions remain, in particular the mechanism of resistance. Nonetheless, data presented here offers the potential to inform TB control programs and has added significant insight into the understanding of PZA resistance in *M. tuberculosis*.

Future studies

1. Investigate fitness cost of different mutations in *pncA* using more refined techniques, such as competition assays. This will provide better insight into the different effects of the different mutations.
2. Create more *in vitro pncA* mutants to investigate downstream transcriptional and proteomic changes associated with *pncA* mutations/deletions.
3. Investigation of isolates with intermediate or reduced PZA resistance (<100 µg/ml) to understand why this occurs as well as to have clinical outcomes data.
4. To expand to the number of isolates tested using the LATE-PCR technique, so that more mutations in *pncA* can be investigated.
5. The LATE-PCR technique needs to be validated on a "raw" specimen type to assess the diagnostic utility for possible implemented of routine DST.
6. Investigation of upstream mutations associated with PZA resistance and the mechanism of action.

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