Genotypic characterization of pyrazinamide resistance in *Mycobacterium tuberculosis* isolated from Lusaka, Zambia

Precious Bwalya[a], Tomoyuki Yamaguchi[b], Georgina Mulundu[a], Chie Nakajima[b, $], Grace Mbulo[c], Eddie Samuneti Solo[c], Yukari Fukushima[b], Kunda Kasakwa[c], Yasuhiko Suzuki[b, $].

[a]The University of Zambia School of Medicine, Lusaka, Zambia

[b]Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

[$]Global Station for Zoonosis Control, Global Institution for Collaborative Research and Education (GI-CoRE), Hokkaido University, Sapporo, Japan

[c]Tuberculosis Laboratory, The University Teaching Hospital, Ministry of Health, Lusaka, Zambia

Corresponding author: Yasuhiko Suzuki

Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Kita 20, Nishi 10, Kita-ku, Sapporo 001-0020, Japan

TEL: +81-11-706-9503, FAX: +81-11-706-7310,

E-mail: suzuki@czc.hokudai.ac.jp

Abstract

Pyrazinamide forms a core part of treatment for all types of tuberculosis (TB) in Zambia. Due to challenges associated with pyrazinamide testing, little information is available to indicate the frequency of resistance to this drug in Zambia. To determine the frequency of pyarazinamide (PZA) resistance and its correlation with mutation in *pncA* in *Mycobacterium tuberculosis* isolated from patients in Lusaka, Zambia, BACTEC MGIT M960 was used for phenotypic PZA susceptibility testing while sequencing was used to determine resistance-conferring mutations in the *pncA.*

Of the 131 isolates analyzed, 32 were phenotypically resistant to PZA. Among multidrug-resistant (MDR) *M. tuberculosis* isolates, the frequency of PZA resistance was 21 of 35 (58.3%). And 27 of 32 PZA resistant isolates had mutations in the *pncA* that seem to confer resistance. With BACTEC MGIT 960 as the reference standard, gene sequencing showed 84.4% sensitivity and 100% specificity. Nine new mutations were identified and the single nucleotide substitution T104G and C195T were the most frequent mutations. However, they were observed in both susceptible and resistant strains and indicating that they are non-resistance conferring mutations.

This study has demonstrated that PZA susceptibility testing is necessary especially in patients suffering from MDR-TB as approximately half of the patients have PZA resistant TB. Similar studies will have to be carried out in other provinces to get an accurate estimate of PZA resistance in Zambia. Mutations in *pncA* were the major mechanism of PZA resistance with no involvement of *rspA* and *panD* genes. However, the presence of mutations among phenotypically PZA susceptible *M. tuberculosis* isolates makes it challenging to independently use genotyping method for the determination of PZA resistance.

Key words: pyrazinamide, *pncA*, resistance, mutation, BACTEC MGIT 960

Introduction:

Tuberculosis has remained a public health concern despite the downward global trend of incidences. It is reported to have topped HIV as a leading cause of death by a single infectious agent with an estimated 1.3 million deaths having occurred in 2016[1] . The emergence of drug resistance is even of greater concern globally because it threatens to roll back the achievements made in halting and reversing tuberculosis.

For the treatment of drug susceptible tuberculosis, isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) are recommended. Pyrazinamide also forms a key component of drugs recommended for the management of MDR-TB. This is due to its ability to act against *Mycobacterium tuberculosis* that persists in acidic environments created during acute inflammation[2]. To exert its bactericidal effect, PZA must be converted to its active derivative pyrazinoic acid by mycobacterial enzyme pyrazinamidase encoded by *pncA*. Pyrazinoic acid has been shown to bind to 30S ribosomal protein S1 (RpsA) preventing trans-translation a process necessary for the survival of mycobacterium in stressful inflammatory environment[3].

Despite its wide use in TB treatment, susceptibility testing of PZA is not routinely performed and WHO does not recommend its inclusion in routine testing owing to the challenges related with phenotypic drug susceptibility testing methods [4]. However, due to the potential synergistic effect PZA has when combined with novel anti-TB drugs, it has become pertinent to understand the resistance patterns to *M. tuberculosis* to PZA. In this context, WHO recently recommended the use of non-radiometric BACTEC M960 in combination with genotypic testing for determination of PZA resistance [5].

The non-radiometric BACTEC M960 is noted for its lack of reproducibility in PZA testing. This has been attributed to the large inoculum size needed for this test, minimum inhibitory

concentration (MIC) of PZA and the acidic media, which inhibits the growth of mycobacteria [6][7]. Nonetheless, it is a better proxy test method for the screening of PZA resistance as compared to the conventional radiometric BACTEC 460 which is labor intensive and the Wayne method which has been shown to be less sensitive due to the varying mechanisms of PZA resistance [8][9]. Several studies have demonstrated that this test method when used in combination with genotypic analysis is a good predictor of PZA resistance [10].

The genotyping method currently being used for PZA testing involves sequencing of the *pncA*, which encodes the pyrazinamidase enzyme. Mutations in this gene have been shown to correlate with PZA resistance phenotype [10]. On the other hand, a small proportion of isolates with this phenotype do not harbor mutations in this gene, suggesting the existence of other mechanisms of resistance. Studies have suggested that efflux of the drug, mutations in *rpsA* gene encoding ribosomal protein S1; and *panD* gene encoding aspartate decarboxylase involved in biosynthesis of pentothanate and coenzyme A are the alternative mechanisms by which mycobacteria acquire PZA resistance [3][11][12]. These mechanisms' significant contribution to PZA resistance is not yet fully established and no method for routine determination of POA efflux yet exists, leaving *pncA* as the principle target for the genotypic PZA susceptibility testing [13][14].

In Zambia, the recent tuberculosis prevalence survey conducted in 2014 revealed that tuberculosis burden was actually higher than was previously estimated with an excess of approximately 4,000 incidences [15]. Multi-drug resistance was detected in an estimated 0.3% and 8% of new cases and recurrent cases respectively [15]. But with drug susceptibility testing (DST) being performed at only three health facilities countrywide and only in suspected treatment failure, there is a strong probability of underestimating the impact of drug resistance in Zambia [16]. Pyrazinamide is an essential component of the standard regimen used in

management of TB elsewhere and in Zambia [45]. While many countries' description of the extent of pyrazinamide resistance is based on research, this kind of information is unavailable in Zambia.

The purpose of this study was to determine the level of PZA resistance among *M. tuberculosis* isolates from patients with pulmonary tuberculosis in Lusaka, the capital city of Zambia. In addition, this study determined characteristics of mutations in *pncA, rspA* and *panD* in relation to PZA resistance.

Materials and Method

Study setting and design

This study was carried out at University Teaching Hospital TB Reference Laboratory in Lusaka, Zambia, which is one of the 3 facilities offering culture and DST for tuberculosis in the country. The laboratory mostly cultures samples from suspected treatment failure. Permission from Hospital Management and Ethical clearance from the University of Zambia biomedical research ethics committee for this study were obtained.

Bacterial isolates.

M. tuberculosis strains isolated from patients with pulmonary tuberculosis between August, 2013 and September, 2015 were used in this study. A total of 131 stored isolates with first line DST results were revived and used for the study. The isolates were cultured in Mycobacterial Growth Indicator Tubes (MGIT, Becton Dickinson & Co., Franklin Lakes, NJ) and incubated in an automated non-radiometric BACTEC MGIT 960 system (Becton Dickinson & Co.) according to manufacturer's instructions. Positive tubes were inoculated on blood agar and incubated for 48 hours to check their purity.

PZA susceptibility testing

BACTEC MGIT 960 PZA kit (Becton Dickinson & Co.) was used for PZA susceptibility testing. The test was performed within 1 to 5 days of instrument positivity of sub-cultured isolates. Two MGIT tubes were used for each isolate with one tube being the Growth control (GC) and the other containing PZA with the final concentration of 100 µg/ml. Prior to inoculation, 0.8 ml of PZA supplement was added to both tubes and there after 100 µl of re-suspended PZA was added. The growth control was then inoculated with 500 µl positive specimen that had been diluted with normal saline in the ration 1:10. The inoculum for the drug containing tube was used directly if suspension was within days 1 and 2 and diluted with normal saline if within 3 to 5 days of instrument positivity. For quality control, H37Rv and a laboratory isolate identified as *M. bovis* were used to control for the susceptible and resistant strains, respectively.

DNA extraction, PCR amplification and sequencing

The contents of positive MGIT (1.5 ml) were heated at 95^{o}C for 30 minutes and DNAs were recovered in the supernatant. The primers used to amplify and sequence the 561 bp *pncA* gene and its promoter region was: *pncA*-f (5’ GCACCAAGGCCGCGATGACAC 3’) and *pncA*-r (5’ CGCGCGTCACCGGTGAACAACC 3’). The 20 µl of PCR amplification mixture contained 10.9 µl of distilled water, 4 µl of 5xGoTaq buffer (green), 0.2 µl of 25 mM dNTP, 0.8 µl of 25 mM $MgCl_2$, 2 µl of 5M betaine, 0.5 µl of 10µM of each primer, 0.1 µl GoTaq (Promega Co., Madison, WS, USA) and 1µl of sample DNA. The amplification of *pncA* was conducted in an iCycler (BioRad, Hercules, CA, USA) under the conditions of: pre-denaturation at 96°C for 1

minute; 35 cycles of denaturation at 96°C for 10 seconds; annealing at 52°C for 10 seconds and extension at 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. DNA sequences of amplified fragments were analysed using a BigDye® terminator version 3.1 cycle sequence reaction kit and an ABI 3130 genetic analyser (Applied Biosystems, Foster, CA, USA) in accordance with the manufacturer's instructions. The obtained sequences were compared to the standard sequence from *M. tuberculosis* H37Rv using Bioedit software. For samples that showed resistance in PZA susceptibility testing, *rpsA* and *panD* sequences were also sequenced according to methods described in previous studies [11][14][17].

Spoligotyping

Spoligotyping was performed as described by Kamerbeek et al (1997) [18]. Briefly, a PCR-based reverse hybridization method was used in which the direct repeat (DR) region of *M. tuberculosis* gene was amplified using a pair of primers. The PCR products were hybridized to a set of 43 oligonucleotide probes corresponding to each spacer covalently bound to the membranes. Obtained spoligo-patterns were then compared to SITVIT data base (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE) for identification of spoligo families and spoligotype international type (SIT) [19].

Statistical analysis

All statistical analyses were performed using SPSS software and significance level was set at 95%. With BACTEC MGIT 960 results as the gold standard; the sensitivity, specificity and the positive and negative predictive value of mutations in *pncA* were calculated.

Results:

PZA resistance

A total of 131 *M. tuberculosis* strains previously isolated from patients with pulmonary tuberculosis was used for this study. Demographic data of the isolates could not be analyzed as the information was incomplete in the laboratory information system. A total of 32 (24.4%) isolates were found resistant to PZA. Drug susceptibility testing for these isolates was repeated to confirm the resistance. Of the 32 PZA resistant isolates, resistance was reproduced in 21 isolates, while 2 isolates were susceptible on repeat test. Results for 9 isolates could not be obtained and these were excluded bringing the total number of isolates analyzed to 122. The results obtained after repeat testing were considered as true phenotype.

The drug susceptibility profiles of isolates showed that 32 (26.2%) were MDR-TB, while 90 (73.1%) were non-MDR TB as shown in table 1. Among MDR-isolates, 16 (50%) isolates were phenotypically resistant to PZA as compared to 5 (5.6%) among the non-MDR-TB *(P value<0.0001)*. Pyrazinamide mono resistance was seen in eight isolates in first PZA testing but on repeat, four isolates had PZA mono resistance phenotype. When PZA resistant isolates were stratified into new and recurrent cases, it was observed that 1(5.6%) isolate was a new case while 17 (94.4%) isolates were from recurrent cases (Table 2). Treatment history of 3 isolates could not be ascertained because of incomplete information in the laboratory information system.

Mutations in pncA, rpsA and panD

Sequencing of *pncA* gene and its promoter region revealed a diversity of mutations in 40 of the 123 total isolates. The most dominant were point mutations. The mutations were well spread along the gene with deletions and insertions clustering towards the downstream (Table 3). Eighteen types of mutations were exclusively identified in the 19 PZA resistant isolates. Two isolates had mutations in the promoter region and both of them exhibited PZA resistant

phenotype. Insertions and deletions were observed in 5 isolates. Six mutations not previously described were identified in this study. Three types of mutations were observed in both susceptible and resistant isolates. Two of these mutations, a synonymous C195T (S65S) and non-synonymous T104G (L35R) mutations were the most frequently observed mutations being identified in 17 and 7 isolates, respectively. The other mutation observed in both resistant and susceptible isolates was A128C (H43P)**.**

To check contributions of mutations in the other genes to PZA resistance, *rpsA* and *panD* sequences of the initial 32 PZA-resistant isolates were also read. It was confirmed that neither *rpsA* nor *panD* in those isolates had a mutation.

To check mutations and lineage specificity, spoligotyping results were obtained all isolates with mutations. Table 3 shows profile of *pncA* gene mutations from the initial PZA testing. Spoligotyping of isolates with C195T revealed that 1 isolate belonged to the CAS1_DELHI (SIT 25) lineage while 16 isolates of this mutation belonged to CAS_KILI (SIT21) lineage. All isolates having the T104G mutation belonged to T1 clade (SIT 53). Aside from these two non-resistances conferring mutations, there were no other mutations that were specific for any genotype.

Correlation between phenotype and genotype

Comparison of phenotyping and genotyping results revealed significant correlations ($p < 0.0001$). The ability of *pncA* sequencing to predict PZA resistance was determined and the sensitivity and specificity was 95.2% (95%CI: 74.1-99.8%) and 99% (95%CI: 93.8-99.9%), respectively (Table 4). The positive predictive value (PPV) and negative predictive value (NPV) were 95.2% and 99%, respectively. Using the results for the first PZA testing, *pncA* sensitivity

and specificity were 84.4% and 100%, respectively. Repeat of PZA testing significantly improved the sensitivity of *pncA* analysis.

Discussion

The critical role of pyrazinamide in TB treatment is undeniable. Its unique characteristic of sterilizing mycobacteria in acid environment led to the current short term treatment therapy and continues to bear on treatment outcome in the hunt for novel anti-TB drugs [20][21][22]. Despite its important role, there are reports of resistance from different parts of the world with rates ranging from 6% in drug susceptible TB to 73% in MDR-TB [23][24]. This study is the first to report on pyrazinamide resistance and its association with *pncA* gene mutations in Zambia.

Pyrazinamide resistance in MDR-TB was 50% and 5.8% in susceptible isolates in this study. These results are similar with those obtained in South Africa where researchers found 52.1% of MDR and 10.2% of fully susceptible isolates were resistant to PZA [25]. A study carried out in Sub-Saharan Africa in rifampicin resistant isolates found 52% and 57% PZA resistance in Ivory Coast and Mali, respectively [26]. In Taiwan and Thailand similar resistance rate in MDR-TB were observed; 54.5% and 49%, respectively [27][23]. In Democratic Republic of Congo (DRC) and Russia however, resistance was higher with 72% of rifampicin resistant isolates and 74.3% of MDR-TB being resistant to PZA [26][24]. Evidently, approximately half of the MDR-TB patients are resistant to pyrazinamide and this is a wide observation in many regions. Interestingly however, there is a marked difference of levels in MDR-TB even within similar geographical locations. While these maybe due to the differences in methodologies, they signify the importance of routine surveillance of PZA resistance. There is also an urgent need for effective treatment regimens tailored to PZA resistant TB.

Analysis of results based on treatment history revealed that PZA resistance was significantly associated with recurrent TB treatment (22.4%) than with new treatment cases (2.4%) (*P value =0.009).* Higher rates of PZA resistance were also reported in retreatment cases in Mozambique's Manhica district; 13.2% in retreatment cases as compared to 1.3% in new cases [28]. A different phenomenon was observed in DRC and Rwanda with no significant difference in PZA resistance between new cases and retreatment cases. Resistance to PZA in these two countries in new cases was 75% and 69%; and in retreatment cases 69% and 67% respectively [26]. The reasons for this difference may be technical or methodological.

Some studies have reported a significant correlation between PZA resistance and mutations in *pncA*. In South Africa, Bishop et all reported that 66.7% of PZA resistant isolates had mutations in *pncA* gene [29]. This correlation was even higher in reports from Canada (89.8%) and South Korea (85.1%) [30][31]. In line with these later reports, the current study found a significant association (95.2%) between PZA resistance and resistance conferring *pncA* gene mutation (X^2 – 101.9, *p value* <0.0001). This high correlation indicates that mutations in *pncA* gene can be exploited for reliable prediction of PZA resistance unlike the unreliable phenotypic testing. Overall, mutations were highly diverse and scattered along the gene, a phenomenon that has been widely observed in various studies [23][27]. The cause of this high diversity has not yet been understood, but de Hertog hypothesized that it points to the low transmissibility of PZA mutant strains as evidenced by few strains having similar mutations [32]. This though would contrast the phenomenon observed in Canada where a mono-resistant strain with similar deletion patterns was isolated from a number of patients [32]. Despite insignificant clustering, some mutations were seen in regions close to amino acid residues known for the catalytic (8, 96, 138), Iron (49, 51, 57, 134) and the substrate (13, 68) binding functions of PZase enzyme [32]. These

mutations may be affecting the enzymes` activities leading to resistance. Most mutations had been previously reported but, a total of 6 new mutations were identified in this study.

A SNP at 195(C-T) was the most prevalent of the mutations observed in a total of 17 isolates followed by a non-synonymous mutation at 104 (T-G) seen in 7 isolates. These mutations were observed in both susceptible and resistant strains. Feuerriegel and other researchers reported that C195T mutation was a genetic marker for CAS_DELHI lineage [33][34]. Contrary to these reports, Spoligotyping of isolates with C195T mutation showed that 16 isolates belonged to CAS1_KILI lineage (SIT 21) while 1 isolate belonged to CAS_DELHI lineage (SIT 25). This mutation maybe a genetic maker for some members of the CAS family and this suggestion is supported by another study that observed it in isolates belonging to two lineages (CAS2 and CAS_DELHI) [35]. All 7 isolates with the non-synonymous T104G mutation belonged to the T1 clade (SIT 53). Therefore, this point mutation may also be a genetic maker of some T1 family. The non-resistance conferring mutations need further studies to explore and determine their implication. In this study the two mutations were considered as non-resistance conferring and were not included in the determination of the correlation between phenotypic resistance and mutations in the *pncA* gene.

The sensitivity of *pncA* gene sequencing for PZA susceptibility testing was found to be 95.2%. This was higher than what was described in a review by Ramirez-Busby et al [10] in which the average sensitivity of *pncA* gene sequencing was 80% and specificity was 91%. Specificity in this study was 99.0%. The sensitivity of *pncA* mutations in predicting PZA resistance in MDR-TB was 100% (16 out of 16). Both parameters in this study were calculated after filtering out the two non-resistance conferring mutations (T104G and C195T). After filtering out the two SNPs, there remained one PZA resistant isolate with wild type *pncA* gene. The lack of mutations in

pncA gene of these isolates indicates an alternative mechanism of resistance such as efflux of the drug or the newly described bifunctional enzyme Rv2783c [36] but not *rspA* and *panD* because no mutations were seen in these mutations in this study.

Repeat susceptibility testing with BACTEC M960 on PZA resistant isolates reproduced 21 resistant isolates, while 2 isolates were susceptible on second attempt. Results for the remaining 9 isolates could not be obtained on second attempt. Of the 2 susceptible isolates, 1 had no mutations in *pncA* gene and this could suggest a true susceptibility result. The other had a resistance conferring mutation (A128C). The later also appeared to be a mixed infection because sequencing results of *rpoB* and *katG* showed 2 peaks in both of these sequences (unpublished data). These results confirm the challenges of reproducibility associated with phenotypic PZA testing using BACTEC M960 PZA reported by other researchers such as Aono and Piersimoni [37][38]. This could be attributed to technical errors or the inherent challenges associated with this method leading to false resistance. This cannot over emphasize the importance of using on point mutations to determine pyrazinamide resistance. .

Conclusion

This study has demonstrated a high prevalence of pyrazinamide resistance in Lusaka in MDR-TB. Consequently, it would be prudent to perform susceptibility testing before adding PZA to MDR-TB therapy. Mutations in *pncA* were the major mechanism of PZA resistance in Lusaka isolates rather than *rspA* or *panD* genes. Nonetheless, some of the mutations were non-resistance conferring with one phenotypically resistant isolate having a wildtype gene suggesting an alternative mechanism of resistance. Identification of non-resistance conferring mutations and filtering of these out has been demonstrated to improve the predictability of PZA resistance using

gene analysis. Phenotypic method cannot be relied upon entirely for PZA resistance because of false resistance results. Genotyping is the best proxy for determining PZA resistance.

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Table 1: Phenotypic Drug Susceptibility profiles of the isolates using second round of PZA susceptibility

Drug resistance pattern	PZA-Resistant	PZA-Sensitive	Total
SHRE	5	2	8
SHR	5	9	14
HRE	3	3	6
HR	2	2	4
SHE	-	1	1
SH	-	1	1
SR	-	2	2
E	-	2	2
S	-	1	1
H	1	12	13
R	0	1	1
Susceptible	4	65	69
Total	21	101	122

S-Streptomycin, H-Isoniazid, R-Rifampin, E-Ethambutol. Susceptible-not resistant to any drug

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Table 2: PZA resistance occurrence in new and recurrent cases

Drug resistance pattern	New cases; no.(%)	Recurrent cases; no.(%)	Unknown	Total
SHRE	0	4	2	6
SHR	1	4		5
HRE	0	1		1
HR	0	2		2
H	0	1		1
R	0	0		0
Susceptible	0	5	1	6
Total	1(5.6%)	17(94.4%)	3 (14.3%)	21

S-streptomycin; H- isoniazid; R-rifampicin; E- ethambutol

Definition: New cases: newly diagnosed and have been treatment for less than 2months

Recurrent treatment case: cases with resistance to previous drugs and relapses.

471 Table 3: profile of *pncA* mutations

Mutation site	Nucleotide mutations	Amino acid change	Number of Isolates (frequency %)	SIT #	Spoligotype lineage	1st PZA susceptibility (# of isolates)	2nd PZA susceptibility	Mutation previously reported?
-7	T→C	N/A**	2 (1.5)	137	X2	R(2)	R;NR	Yes [39]
29	A→G	Q10R	1 (0.8)	2173	LAM11_ZWE	R	NR	Yes [40]
104#	T→G	L35R	6 (4.6)	53	T1	R(2); S(4)	S(5);NR	Yes [41]
128	A→C	H43P	1 (0.8)	52	T2	R	R	Yes [41]
128	A→C	H43P	1 (0.8)	53	T1	R	S(mxd)	Yes [41]
146	A→G	D49G	1 (0.8)	New	orphan	R	R	Yes [41]
146	A→G	D49G	1 (0.8)	New	orphan	R	R	Yes [41]
151	C→G	H51D	1 (0.8)	34	S	R	NR	Yes [41]
160	C→T	P56S	1 (0.8)	42	LAM9	R	R	Yes [41]
195#	C→T	S65S	13 (10.7)	21	CAS1_KILI	R; S(12)	R; S(12)	Yes [41]
195#	C→T	S65S	1 (0.8)	25	CAS_DELHI	S	S	Yes [41]
214	T→C	C72R	1 (0.8)	New	orphan	R	R	Yes [41]
238*	G→A	D80N	1 (0.8)	59	LAM11_ZWE	R	R	No
254	T→C	L85P	1 (0.8)	New	orphan	R	NR	Yes [41]
290*	G→C	G97A	1 (0.8)	59	LAM11_ZWE	R	NR	No
313	G→C	G105R	1 (0.8)	53	T1	R	R	Yes [42]
357	G→T	W119C	1 (0.8)	59	LAM11_ZWE	R	R	Yes [43]
373*	deletion GTCGATGAG	Frameshift	1 (0.8)	59	LAM11_ZWE	R	R	No
383	T→G	V128G	1 (0.8)	New	Orphan	R	R	Yes [41]
389	Insertion GG	Frameshift	1 (0.8)	59	LAM11_ZWE	R	R	Yes [26]
394*	Deletion G	Frameshift	1 (0.8)	815	LAM11_ZWE	R	R	No
410*	Insertion TG	Frameshift	1 (0.8)	815	LAM11_ZWE	R	R	No
460	A→G	R154G	2 (1.5)	59	LAM11_ZWE	R(2)	R;NR	Yes [31]
512	C→T	A171V	1 (0.8)	59	LAM11_ZWE	R	R	Yes [41]
195#; -11	C→T; A→C	S65S; N/A	1 (0.8)	21	CAS1_KILI	R	R	Yes [44]
104#; 217*	T→G; G→T	L35R; V73F	1 (0.8)	53	T1	R	R	No
195#; 22	C→T; G→A	S65S; D8N	1 (0.8)	21	CAS1_KILI	R	R	Yes [39]
195#; 129*	C→T, Insertion CG	S65S; Frameshift	1 (0.8)	21	CAS1_KILI	R	R	No
Wild type		wild type	84 (64.1)		N/A			N/A

*New mutations; #Non-resistance conferring mutations; N/A- Not applicable; NR- No results; mxd- mixed infection.

R- Resistance; S- susceptible

Table 4: Diagnostic value of mutation detection for PZA susceptibility testing

PZA resistant		PZAsusceptible		Sensitivity% (95%CI)	Specificity% (95%CI)
with mutation	without mutation	with mutation	without mutation		
20	1	1	100	95.2 (74.1-99.8%)	99.0 (93.8-99.9%)