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Original article

Detection of pyrazinamide resistance of *Mycobacterium tuberculosis* using nicotinamide as a surrogate

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ABSTRACT

Objectives: Despite the importance of pyrazinamide (PZA) in tuberculosis treatment, PZA susceptibility testing is not routinely performed because of its acid pH requirement. We evaluated the Microplate Alamar Blue assay (MABA) to detect resistance to PZA using nicotinamide (NIC) as a surrogate in neutral pH and identify the appropriate cutoff point for the assay.
Methods: The NIC minimal inhibition concentrations (MICs) for 125 *Mycobacterium tuberculosis* clinical isolates were tested by MABA at nine different concentrations (8–2000 μg/mL). The PZA susceptibility testing by the BACTEC MGIT 960 system was used as a reference method. The *pncA* gene and its promoter region were sequenced for all the recruited strains.
Results: A total of 64 of 125 clinical isolates were identified as resistant by MGIT 960. Using a minimum inhibitory concentration (MIC) of >500 μg/mL as the cutoff concentration to define resistance presented the best fit of the MABA assay with the MGIT 960 outcomes. MABA demonstrated sensitivity of 100% (95% confidence interval, 92.6–100), specificity of 95.2% (95% confidence interval, 86.0–98.8) and an accuracy of 97.6% compared to the MGIT 960 method. Nine PZA susceptible strains defined by MGIT 960 also had *pncA* mutations; however, three of them were defined as PZA resistant by NIC MABA with MIC ≥2000 μg/mL.
Conclusions: The NIC substitution method for PZA susceptibility test is reliable, cheap, rapid and easy, which makes it promising for use in clinical laboratories. **Y. Hu, Clin Microbiol Infect 2017;23:835**

Introduction

Pyrazinamide (PZA) is the backbone of the short-course chemotherapy of tuberculosis (TB) as a result of its unique ability to kill the semidormant tubercle bacilli that reside in acidic inflammatory environments. Because of its effectiveness and low price, PZA is widely used in the intensive phase of anti-TB treatment and recently has become pivotal in multidrug-resistant (MDR) TB, defined as resistance to at least isoniazid and rifampicin treatment [1]. In recent years various studies have reported PZA resistance among MDR and non-MDR TB patients, which highlights the importance of drug susceptibility testing (DST) before the drugs are administered to patients [2–4].

However, detection of PZA susceptibility is not common compared to other anti-TB drugs. Lack of PZA DST in many regional labs in site such as Eastern Europe and Asia may be due to the use of solid media DST, which is not recommended for PZA. PZA is active only in an acidic environment (optimal pH, 5.5); it thus affects the growth of mycobacteria, making the test unreliable. It has been reported that some factors can easily affect the accuracy of the test, such as inoculum size, components of the media and age of the cells [5–7]. Although the BACTEC MGIT 960 system (BD Biosciences, Sparks, MD, USA), which uses special acid liquid medium, has become more popular for the detection of PZA resistance,

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expensive equipment and reagents limit its widespread adoption. Molecular testing for drug resistance detection had been used in TB for many years. Although a good correlation had been established between PZA resistance and *pncA* mutations, the highly diverse and widely scattered characterizations of the mutations throughout the 561 bp long gene and its regulatory region make this commonly used technique unfeasible. Moreover, not all PZA-resistant *M. tuberculosis* isolates have mutations in their *pncA* genes, while some *pncA* mutation types cause very low-level PZA resistance which may have negligible clinical significance. A rapid, simple, reliable and cheap PZA susceptibility testing method is urgently needed.

PZA is a prodrug. It is converted to its active form by pyrazinamidase (PZase). Most PZA-resistant strains have mutations in *pncA*, the gene encoding PZase, leading to enzyme activity loss or reduction. Nicotinamide (NIC) is a structural analogue of PZA [8] which possesses anti-TB activity and is also converted to its active acid form by PZase [9]. Studies have found that TB strains resistant to PZA were also resistant to NIC; however, the NIC inhibition test could be performed at neutral pH which did not hinder mycobacterial growth. Martin et al. [10] developed a colourimetric method, the resazurin microtitre assay, using NIC as a surrogate for PZA to avoid the need for acidification of the medium. Martin et al. [11] also developed a nitrate reductase assay using NIC in Lowenstein-Jensen medium at neutral pH. Both methods were reported to be reliable, cheap, rapid and promising. However, only one study has been reported since then to repeat the nitrate reductase assay and the colourimetric methods but with MGIT 960 tubes [12].

The objective of our study was to apply Microplate Alamar Blue assay (MABA) to detect PZA resistance using NIC at neutral pH. We compared these results with BACTEC MGIT 960 method and *pncA* gene sequencing to evaluate the NIC substitution method and the cutoff point recommended by others.

Materials and methods

M. tuberculosis isolates

A total of 125 clinical *M. tuberculosis* isolates were assessed. To ensure enough PZA-resistant strains, 70 MDR TB isolates were selected from the mycobacterial collection of the Beijing Chest Hospital, while the other isolates were obtained continuously from routine mycobacterial cultures. The laboratory strain H37Rv (ATCC 27294), which is susceptible to PZA, and the bacillus Calmette-Guérin (BCG) strain (ATCC 34540), which is resistant to PZA naturally, were used as controls.

MABA

NIC (Sigma-Aldrich, Steinheim, Germany) stock solution was prepared at 200 mg/mL concentration in distilled water, filter sterilized and stored at 4°C for no more than 3 days. Alamar Blue (Thermo Fisher, Waltham, MA, USA) working solution was a mixture of Alamar Blue and 5% Tween-80 (mixing ratio, 5:2). The MABA was performed as described by Cho et al. [13]. Briefly, the inoculum was prepared from fresh colony on Lowenstein-Jensen medium in saline, adjusted to a turbidity equivalent to that of a McFarland no. 1 standard and then diluted (1:25) in Middlebrook 7H9-S broth (BD Biosciences), then inoculated at 200 μL suspension in each well. For each isolate, the tested concentrations of NIC included 8, 16, 32, 63, 125, 250, 500, 1000, 1500 and 2000 μg/mL. The plate was covered, sealed in a plastic bag and incubated at 37°C. After a week's incubation, 70 μL of fresh Alamar Blue working solution was added to each well, and the plate was then reincubated overnight. A change in colour from blue to pink indicated the growth of bacteria. Minimum inhibitory concentration (MIC) was defined as the lowest concentration that prevented this change in colour. Quintuple assays were conducted with the BCG and H37Rv laboratory strains to evaluate the reproducibility of the NIC MIC test.

PZA susceptibility testing by BACTEC MGIT 960

The phenotypic PZA susceptibility testing was performed by BACTEC MGIT 960 system with MGIT 960 PZA kits (BD Biosciences) following the manufacturer's instructions but with reduced inoculum. Instead of 0.5 mL cell suspension, 0.25 mL was used in the PZA susceptibility testing. The critical concentration of PZA used in this method was 100 μg/mL, and the BCG and H37Rv laboratory strains were used as PZA-resistant and PZA-susceptible controls respectively.

DNA sequencing of pncA gene plus its promoter region

The *pncA* gene plus its promoter region was amplified and sequenced for the recruited isolates with the following primers: *pncA*-F: 5′-GTCATGGACCCTAT ATCTGTGGCTGCCG CGTCG-3′ and *pncA*-R: 5′-TCAGGAGCTGCAAACCAACTCGACGCTGG-3′. The DNA preparation and PCR amplification were performed as described previously [4]. PCR products were purified and sequenced by Ruibo BioTech (Beijing, China). DNA sequences were aligned with the homologous sequences of the reference strain *M. tuberculosis* H37Rv.

Statistical analysis

Statistical analysis was performed by SPSS 12.0 (IBM SPSS, Chicago, IL, USA). The sensitivity, specificity and accuracy of MABA assay to detect PZA resistance in MDR TB were verified and evaluated compared to the BACTEC MGIT 960 method and *pncA* gene sequencing, and were calculated using an online tool (http://vassarstats.net).

Results

Tentative cutoff point establishment using MGIT 960 method as a reference

Sixty-one and 64 isolates were defined as resistant or susceptible strains respectively by the MGIT 960 PZA susceptibility testing method. Cutoff point definition was based on the best fit of the MABA assay with the MGIT 960 outcomes. If all strains having MICs of NIC higher than 500 μg/mL were considered as PZA resistant, the sensitivity and specificity for MABA assay were 100% (95% confidence interval, 92.6–100) and 95.3% (95% confidence interval, 86.0–98.8) respectively. If the 500 μg/mL point was considered to be resistant, the sensitivity was still 100% but the specificity decreased to 78.1%. Therefore, we used >500 μg/mL as a tentative cutoff point for further analysis.

Comparison of PZA susceptibility testing outcomes between MGIT 960 and NIC MABA

For the MABA assay, results were available after an average of 9 days; for the BACTEC MGIT 960 method, results were available after an average of 10 days. The MGIT 960 and NIC MABA outcomes for the 125 recruited isolates are compared in Table 1.

From the 61 PZA-resistant strains, determined by MGIT 960, 49 (80.3%) had NIC MICs of ≥2000 μg/mL, 8 (13.1%) had MICs of 1500 μg/mL and 4 (6.6%) had a MIC of 1000 μg/mL. Among the isolates categorized as sensitive by MGIT 960, three strains showed

Table 1
Comparison of NIC MABA with MGIT 960 method for pyrazinamide susceptibility testing among 125 tuberculosis isolates

MGIT 960	NIC MABA	
	Resistant	Susceptible
Resistant	61	0
Susceptible	3	61

Sensitivity = 61/61 × 100% = 100%; specificity = 61/64 × 100% = 95.3%; accuracy = 122/125 × 100% = 97.6%.
MABA, Microplate Alamar Blue assay; NIC, nicotinamide.

discrepant NIC outcomes with MICs of ≥2000 μg/mL, ten (15.6%) had MICs of 500 μg/mL, while the others (95.3%) had MICs of ≤250 μg/mL (Table 2). Both the MGIT 960 and MABA methods were repeated with all the discrepant isolates, which yielded concordant susceptibility outcomes. Although direct MIC value comparison demonstrated minor deviations between different tests, which is a general drawback for microtitre plate assay [13,14], most of the deviations appertained to a narrow scope within one double dilution concentration discrepancy, and those deviations were bidirectional. Quintuple NIC assays for BCG strain obtained uniform outcomes with MICs of >2000 μg/mL, and MICs for H37Rv were 250 μg/mL four times and 125 μg/mL once.

pncA gene mutation

The *pncA* gene plus promoter region of all the recruited strains was sequenced; outcomes are listed in Table 2. Forty-eight (78.7%) of 61 of the PZA-resistant strains (defined by MGIT 960) had mutations within their *pncA* gene plus promoter region. Among these 48 *pncA*-mutated strains, 43 had *pncA* mutations resulting in amino acid substitutions, two had nucleotide insertion and three had nucleotide deletion, which all caused frameshifts. However, nine MGIT 960–defined PZA-susceptible strains also had *pncA* mutations, which all led to amino acid substitutions. Among those nine strains, the NIC MICs are as follows: three strains, ≥2000 μg/mL; three strains, 500 μg/mL; two strains, 250 μg/mL; one strain, 125 μg/mL.

Discussion

Limited availability of the specialized equipment and low pH media requirement render the PZA susceptibility testing in clinical mycobacteriology laboratories more demanding. Reports from different countries demonstrate that PZA resistance rates are approximately 40 to 50% among MDR TB isolates [2–4], and are less frequent but are occasionally seen among non-MDR TB isolates, which highlights the importance of performing PZA DST before a regimen that contains this drug is established.

The new PZA susceptibility testing using NIC as a surrogate for PZA is promising because of the application of neutral pH in the assay. It is well known that the low pH used for PZA susceptibility testing hinders the reproducibility and reliability of the assay. Piersimoni reported a high percentage of false-positive findings with PZA susceptibility testing by MGIT 960 according to manufacturer's protocol [6], which raised concerns for its clinical use. We also found approximately 10% of the outcomes to be inconsistent when tests were repeated using the recommended method, so we reduced the inoculum we used in this assay according to Piersimoni's recommendation to improve the reliability of the MGIT 960 assay. According to the outcomes and our comparisons of the different methods, no false-positive findings were observed.

As a new phenotypic DST method, setting up an appropriate cutoff concentration needs large-scale evaluation and validation. Martin and colleagues chose >250 μg/mL as the cutoff concentration for a resazurin microtitre assay method after assessment with 25 PZA-resistant clinical isolates; the only other NIC test adopted this cutoff concentration but used the MGIT 960 method [10,12]. In this assay, we defined the cutoff concentration as >500 μg/mL according to our MGIT 960 outcomes of 61 PZA-resistant and 64 PZA-sensitive clinical isolates. The sensitivity and specificity for the MABA assay were 100% and 95.3% respectively. However, if we used >250 μg/mL as a cutoff concentration, considering the 500 μg/mL point as resistant, then the sensitivity remained 100% but the specificity decreased to 78.1%. The discrepancy between the cutoff concentrations' definition could mainly reflect the strain variation from different studies; however, it might also demonstrate that the MICs of PZA-susceptible and -resistant strains overlap at around 500 μg/mL for the NIC inhibition test. Setting up the cutoff concentration for anti-TB drugs is generally referenced to the epidemiologic cutoff value which best distinguishes the wild type from all variant strains [15]. To define the epidemiologic cutoff value of NIC, multiple centres and a large quantity of strain involvement are required in the future. In contrast to PZA, the tentative critical concentration for NIC is much higher, demonstrating that although NIC and PZA are analogue drugs and are converted to their active

Table 2
Pyrazinamide susceptibility outcomes by MGIT 960 method, NIC MIC distribution and *pncA* and promoter region sequencing outcomes of 125 isolates

MGIT 960 (no. of isolates)	NIC MABA		*pncA* and promoter region sequencing outcomes (no. of isolates)
	MIC (μg/mL) (no. if isolates)	%	
Susceptible (64)	16 (1)	1.6	WT (1)
	32 (2)	3.1	WT (2)
	63 (3)	4.6	WT (3)
	125 (23)	35.9	WT (22); Asp12Glu (1);
	250 (22)	34.4	WT (22); Ala143Val (1), Val163Ala (1)
	500 (10)	15.6	WT (7); Ile6Thr (1), Val139Leu (1), Vla163Ala (1)
	2000 (1)	1.6	His51Gln (1)
	>2000 (2)	3.1	Asp63Gly (1), Thr76Ile (1)
Resistant (61)	1000 (4)	6.6	WT (2); His51Arg (1), Ala146Thr (1)
	1500 (8)	13.1	WT (4);Trp68Arg (1), Val131Ala (1), Ala146Val (1), Ala171Val (1)
	2000 (5)	8.2	Thr17Phe (1), Thr76Pro (1), Leu159Arg (1), Glu174Lys (1), Met175Thr (1)
	>2000 (44)	72.1	WT (7); A DEL-39 (1), C DEL-31 (1), G INS-27 (1), A -11G (3), Gln10Stop codon (1), Asp12Asn (2), Asp12Ala (1), Val7Phe (1), Leu27Pro (1), Ala28Asp (1), Thr47Ala (1), Pro54Leu (1), His57Try (1), Trp68Arg (1), His71Tyr (1), Thr76Pro (1), Leu85Arg (1), Phe94Leu (1), Lys96Arg (1), GDEL301 (1), Ala102Pro (1), Leu116Arg (1), Trp119Arg (1), Asp136Asn (1), Val139Gly (1), G INS 416 (1), Arg140Gly (1), Thr142Lys (1), Ala146Thr (1), Ala171Val (1), Ser185Gly (2), Ser186Pro (1), Thr114Ala/T-7C (1)

DEL, deletion; INS, insertion; MABA, Microplate Alamar Blue assay; MIC, minimum inhibitory concentration; NIC, nicotinamide; WT, wild type.

forms by same enzyme (PZase), PZA has more potent bactericidal efficacy than NIC.

In our assay, the NIC method presented a high concordance with the controlled PZA susceptibility testing by MGIT 960. A 97.6% accuracy was obtained among the 125 tested strains, which revealed that the NIC MIC method is at least as good as MGIT 960 method for PZA susceptibility testing. NIC MABA was as quick as the MGIT 960 method for isolates, but no expensive equipment and reagents were needed. All the three strains with discrepant outcomes presented MGIT 960–sensitive but NIC MIC–resistant outcomes, while all those strains had *pncA* mutations. We repeated both the phenotypic susceptibility testing and *pncA* sequencing, and obtained consistent outcomes. We presumed that those three strains had more chance to be PZA resistant but were signaled as susceptible by MGIT 960 for some unknown reason, as the reliability of PZA susceptibility testing by MGIT 960 method can be easily affected by different elements [5].

Among the recruited isolates, only 78.7% (48/61) MGIT 960–defined and 79.7% (51/64) NIC-defined PZA-resistant strains had *pncA* mutations, which demonstrated the existence of other PZA resistance mechanisms [13]. Although the NIC MIC test demonstrated higher consistence with *pncA* sequencing (79.7% vs. 78.7%), there was no significant difference ($p > 0.05$). However, 9.8% (6/61) PZA-susceptible strains defined by both MGIT 960 and NIC MIC methods also had *pncA* mutations. These mutations could either be the non-resistance-conferring mutations [16] or could have only minor effects on the activity of PZase, which cause low-level PZA resistance that cannot be detected by both phenotypic methods. The low-level resistance could lead to elevated MICs compared with wild-type strains, but because the MICs were overlapped with the PZA-susceptible strains, such undetected PZA resistance might not cause severe clinical problems [17].

Conclusions

In the strains we studied, a >500 μg/mL cutoff concentration best separated the PZA-susceptible and -resistant strains. The NIC substitution method for PZA susceptibility test is reliable, cheap, rapid and easy to perform, which makes it promising for use in clinical laboratories.

Transparency Declaration

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