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A new rapid and simple colorimetric method to detect pyrazinamide resistance in *Mycobacterium tuberculosis* using nicotinamide

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Objectives: The purpose of this study was to develop and assess a rapid method for pyrazinamide resistance detection in *Mycobacterium tuberculosis* using nicotinamide in a colorimetric resazurin assay.

Methods: We have tested *M. tuberculosis* isolates using nicotinamide in a 96-well format with the redox indicator resazurin (REMA) and compared results using the BACTEC 460-TB system with two concentrations of pyrazinamide (100 and 300 mg/L), as well as the Wayne method for detecting pyrazinamidase activity. Mutations in the *pncA* gene were detected by DNA sequencing of the pyrazinamide-resistant strains.

Results: Out of 95 clinical isolates of *M. tuberculosis* tested, 25 were determined to be resistant by the Wayne, BACTEC (300 mg/L), and the REMA nicotinamide methods. Using a nicotinamide MIC > 250 mg/L as the cut-off for defining resistance, only one strain was falsely labelled as resistant. The REMA nicotinamide assay demonstrated a sensitivity of 100% and a specificity of 98%. The BACTEC (100 mg/L) falsely classified 8 strains as resistant. DNA sequencing detected mutations in 18/22 of the *pncA* genes from pyrazinamide-resistant strains.

Conclusions: The REMA plate using nicotinamide to detect resistance to pyrazinamide is a simple and rapid method that could be useful in limited-resource countries.

Keywords: drug resistance, first-line drug, resazurin, susceptibility testing

Introduction

Pyrazinamide is an important first-line antituberculosis drug used in combination with isoniazid, rifampicin and ethambutol in the short-course treatment regimens recommended by the World Health Organization.[1] Pyrazinamide is not active against *Mycobacterium tuberculosis* under normal culture conditions near neutral pH, but it is active in acid medium (pH 5.5) and kills semi-dormant bacilli that persist in acid pH environments inside macrophages.[2–5] These conditions make *in vitro* drug susceptibility testing (DST) more difficult since poor growth of *M. tuberculosis* isolates is obtained in acid medium.[6–9] Due to this major difficulty, pyrazinamide susceptibility testing is not routinely performed in many laboratories in the world, and comprehensive surveillance studies of pyrazinamide resistance are rare. The radiometric BACTEC 460-TB using the special pyrazinamide acid liquid medium, while considered the most reliable test, and thus the reference method, is too costly to be implemented in low-resources countries. An alternative approach is to assess the presence of active pyrazinamidase (PZAse), which is required for susceptibility to the drug, by detecting the hydrolysis of pyrazinamide into pyrazinoic acid (POA) through a change of colour in the Wayne assay.[10]

In 1945, nicotinamide, an analogue of pyrazinamide, was found to have antituberculosis activity and to be active against *M. tuberculosis* H37Rv *in vitro*.[5,11,12] However it was found later that nicotinamide and isoniazid have antagonism when used together;[13] for this reason, nicotinamide was considered contraindicated for antituberculosis chemotherapy and interest in it declined. In 1972, Brander *et al.* reported a preliminary study

showing that pyrazinamide susceptibility could be reliably determined using high concentrations of nicotinamide incorporated into Löwenstein–Jensen medium (LJ), which was later confirmed by Kalish *et al.*[14,15] However over the past 25 years, no subsequent study has investigated the use of nicotinamide for DST of *M. tuberculosis*. Due to structural similarities, the prodrugs nicotinamide and pyrazinamide are both converted by the *M. tuberculosis* nicotinamidase enzyme, also called PZAse, into their active acid forms, nicotinic acid and POA, respectively. *M. tuberculosis* strains that have lost their PZAse activity, and thus their ability to convert pyrazinamide into POA, and nicotinamide into nicotinic acid, are resistant to both pyrazinamide and nicotinamide.[16] Most pyrazinamide-resistant strains have mutations in *pncA*, the gene encoding the *M. tuberculosis* PZAse.[17–19] However, while *M. tuberculosis* requires acid media (pH ∼5.5) to convert pyrazinamide into POA, it can convert nicotinamide into nicotinic acid at a more physiological pH that does not hinder bacterial growth.

We recently developed a resazurin microtitre assay plate (REMA)[20–23] for rapidly detecting resistance of *M. tuberculosis* to first- and second-line drugs and demonstrated that it correlated very well with the proportion method. This assay is based on the ability of actively metabolizing bacteria to reduce the resazurin producing a change in colour from blue to pink. Using the same assay format, we developed and evaluated a colorimetric method to determine the resistance to pyrazinamide by simply using nicotinamide instead of pyrazinamide, thus avoiding the need for acidification of the medium. In the present study, the MIC of pyrazinamide determined by the nicotinamide REMA plate was compared with resistance results obtained with the BACTEC 460-TB method using two different pyrazinamide concentrations, and also with pyrazinamide susceptibility assessed by the enzymatic Wayne method.

Materials and methods

M. tuberculosis isolates

A total of 95 *M. tuberculosis* clinical isolates, originating from countries with a high prevalence of multidrug-resistant tuberculosis (MDR-TB), were used for this evaluation. *M. tuberculosis* H37Rv, susceptible to pyrazinamide (ATCC 27294), and a pyrazinamide-resistant strain (ATCC 35828) were used as controls.

Drug

Nicotinamide (Sigma Aldrich, Steinheim, Germany) stock solution was prepared at 40 mg/mL in distilled water, filter-sterilized and stored at –20°C until use.

Colorimetric reagent resazurin

A stock solution of resazurin sodium salt (Acros Organic N.V., Geel, Belgium) was prepared at 0.01% in distilled water, filter-sterilized and kept at 4°C for no more than 1 week.

Resazurin assay (REMA)

The REMA plate method was carried out as described by Palomino *et al.*[23] Briefly, the inoculum was prepared from a fresh colony on LJ in 7H9 medium supplemented with 0.1% casitone/0.5% glycerol/10% OADC, adjusted to a turbidity equivalent to that of a McFarland no. 1 standard and diluted 1:10. The range of concentrations tested

for nicotinamide was 8–2000 mg/L. The plate was covered, sealed in a plastic bag and incubated at 37°C in normal atmosphere. After 7 days of incubation, 30 μL of resazurin solution was added to each well, and the plate was reincubated overnight. A change in colour from blue to pink indicated the growth of bacteria, and the MIC was defined as the lowest concentration of the drug that prevented this change in colour.

Wayne method

Testing of PZAse activity was performed according to the Wayne method.[10] Briefly, a heavy loopful of growth from a fresh culture on LJ medium was inoculated onto the surface of two Dubos agar butts containing 100 mg/L pyrazinamide and 2 mg/mL sodium pyruvate prepared in a screw cap tube. The tubes were incubated at 37°C for 4 days, after which 1 mL of ferrous ammonium phosphate solution, prepared just before use, was added to each tube. After 30 min the tubes were examined for a pink band in the agar medium. Negative tubes were refrigerated for 4 h and re-examined. If the reaction was negative after 4 days of incubation, the test was repeated at 7 days using the second tube. The appearance of a pink band, which forms at the surface of the agar and diffuses into the medium, indicates the enzymatic hydrolysis of the pyrazinamide into free POA. A positive result, a pink band in the agar, indicated PZAse activity and such a strain was considered susceptible to pyrazinamide, while a culture showing no pink band colour in the agar was considered resistant to pyrazinamide.

BACTEC 460-TB method

Susceptibility to pyrazinamide was also tested by the BACTEC 460-TB method, according to the manufacturer's instructions (Becton Dickinson Diagnostic Instruments, Sparks, MD, USA), using two concentrations of pyrazinamide. Three culture vials with BACTEC pyrazinamide test medium, pH 6.0, supplemented with polyoxyethylene stearate (POES) were inoculated: one vial without drug was used as control; one contained 100 mg/L pyrazinamide; and the third contained 300 mg/L pyrazinamide. The vials were incubated at 37°C and tested daily on the BACTEC 460-TB instrument. For both concentrations of pyrazinamide used (100 and 300 mg/L) when the growth index (GI) of the control vial reached 200 or more, results were interpreted as follows: if the GI in the drug vial was <9% of the GI in the control vial, the strain was considered susceptible; if >11% the strain was considered resistant; if between 9% and 11% the strain was considered borderline. If a GI of 200 was not obtained within 20 days in the control vial, the test was considered uninterpretable.

DNA sequencing

DNA was obtained by resuspending a loopful of culture into 200 μL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), which was heat inactivated at 100°C for 10 min and then centrifuged (10 000 **g** for 20 min at 4°C). Ten microlitres was used for each PCR. To determine the DNA sequence of the *pncA* gene in the pyrazinamide-resistant strains of *M. tuberculosis*, the *pncA* gene was amplified using the forward and reverse primers P1: 5′-GTCGGTCATGTTCG-CGATCG-3′ (from 105 bp upstream of *pncA*) and P6: 5′-GCTTTGC-CGAGCGCTCCA-3′ (from 60 bp downstream of the stop codon) described by Scorpio *et al.*[19] Cycling parameters were 95°C for 5 min followed by 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; 40 cycles were performed and were followed by a final elongation of 72°C for 10 min. The expected size of the *pncA* PCR products was 720 bp. The PCR product was sequenced with an automatic DNA sequencer using the same primers P1 and P6. Mutations in the

Table 1. Pyrazinamide (PZA) susceptibility of the 95 clinical isolates of *M. tuberculosis* using the Wayne and the BACTEC 460-TB radiometric methods

	Wayne PZAse	BACTEC 460-TB radiometric	
		300 mg/L PZA	100 mg/L PZA
Resistant	25	25	33
Susceptible	70	70	62

sequences of the *pncA* gene from pyrazinamide-resistant strains were identified by comparison with the wild-type *M. tuberculosis pncA* gene sequence[17] using BLAST (www.ncbi.nlm.nih.gov) or MacVector.

Statistical analysis

The MedCalc Software (Mariakerke, Belgium) was used to calculate the cut-off point of resistant and susceptible strains and was also used to calculate the sensitivity (ability to detect true resistance) and the specificity (ability to detect true susceptibility).[24]

Results

With the REMA plate assay, results were available after an average of 8 days of incubation, while with the Wayne method and the BACTEC 460-TB, results were obtained after 7 and 10 days, respectively. Results of the susceptibility testing by the Wayne method and the BACTEC 460-TB are summarized in Table 1. Out of 95 isolates tested, 25 were found to be resistant to pyrazinamide by both the Wayne method and the BACTEC 460-TB when used with a concentration of 300 mg/L pyrazinamide giving a correlation of 100%. However, when a concentration of 100 mg/L of pyrazinamide was used in the BACTEC 460-TB, 33 isolates were determined to be pyrazinamide resistant.

The correlation between nicotinamide MIC results obtained by the REMA plate and results obtained by the Wayne and BACTEC 460-TB methods is shown in Table 2. Of the 25 *M. tuberculosis* isolates determined to be resistant to pyrazinamide by the Wayne and BACTEC 460-TB (300 mg/L pyrazinamide) methods, 18 (72%) had nicotinamide MICs ≥ 2000 mg/L, 6 (24%) had MICs of 1000 mg/L and 1 (4%) had a nicotinamide MIC of 500 mg/L. The majority of isolates judged pyrazinamide susceptible by the Wayne and BACTEC 460-TB (300 mg/L) methods had nicotinamide MICs of 125 mg/L or lower (64/70, 91%), five had an MIC of 250 mg/L and one susceptible isolate was discordant with a nicotinamide MIC of 500 mg/L. The REMA assay was repeated on this isolate with the same result, and sequencing of its *pncA* gene found no mutation.

Figure 1 shows a dot plot diagram of the results that allows the identification of a cut-off point or 'critical concentration' that defines susceptible and resistant strains based on the best fit of the colorimetric results with the conventional method. If all strains having an MIC of nicotinamide higher than 250 mg/L are considered pyrazinamide resistant, the sensitivity and specificity for the REMA nicotinamide test were 100% and 98.6%, respectively; all resistant isolates could be correctly detected. All of the strains resistant to pyrazinamide were also resistant to at least isoniazid and rifampicin (MDR-TB).

Table 2. MIC of nicotinamide by the REMA plate compared with results by the Wayne/BACTEC 460-TB methods for 95 isolates of *M. tuberculosis*

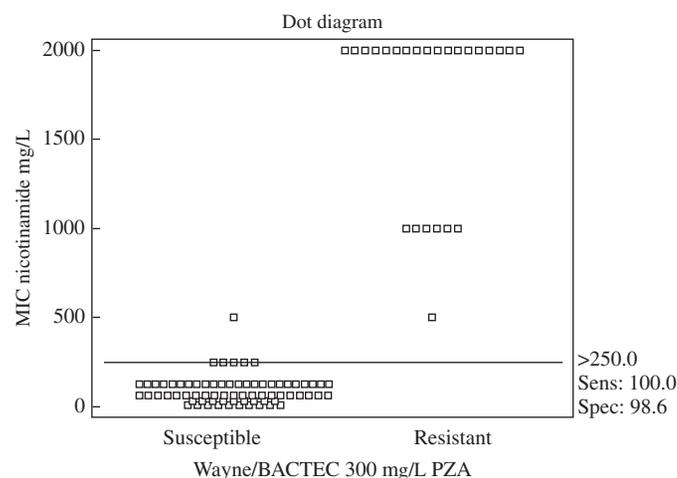| Wayne/ BACTEC 460-TB 300 mg/L (*n* = 95)	No. of isolates with MIC of nicotinamide (mg/L)								
	≤8	16	32	62.5	125	250	500	1000	≥2000
Resistant (*n* = 25)							1	6	18
Susceptible (*n* = 70)	2	8	9	21	24	5	1		

Figure 1. Dot plot diagram of the susceptibilities to pyrazinamide of 95 isolates of *M. tuberculosis* by the Wayne/BACTEC 460-TB methods versus MIC values obtained by the nicotinamide REMA plate.

We also analysed the DNA sequence of the *pncA* gene from 22 pyrazinamide-resistant isolates. Three resistant isolates could not be analysed because of lack of enough material. Mutations in the *pncA* gene were identified by comparison with sequence of the wild-type *M. tuberculosis pncA* gene, and the results are shown in Table 3. Among 22 pyrazinamide-resistant strains analysed, 18 had *pncA* mutations causing an amino acid substitution. These mutations were dispersed throughout the *pncA* gene. Out of the 22 strains confirmed to be pyrazinamide resistant, 4 did not contain *pncA* mutations.

Discussion

Pyrazinamide is a very peculiar drug. It is well known that pyrazinamide has high *in vivo* but poor *in vitro* activity and that the MIC for *M. tuberculosis* could be 10–20 times higher than the attainable serum concentration; even in acid medium, pyrazinamide kills *M. tuberculosis* slowly and incompletely.[8,25]

Because timely detection of drug resistance is essential for the management of MDR-TB, a simple and reliable method for rapid DST is needed, especially in resource-poor settings. In the present

Table 3. *pncA* mutation of pyrazinamide-resistant clinical
M. tuberculosis isolates

Strain	Nicotinamide MIC (mg/L)	*pncA* mutation
99-884	≥2000	Gly-97→Asp
00-742	≥2000	Ser-164→Pro
01-1647	≥2000	Leu-120→Pro
02-684	≥2000	no mutation
02-650	≥2000	Trp-119→Arg
00-351	≥2000	no mutation
02-154	≥2000	Trp-119→Arg
02-173	≥2000	no mutation
00-856	≥2000	Val-139→Gly
99-1900	≥2000	Lys-111→Gln
00-857	≥2000	Ala-36→Val
99-1896	≥2000	Asp-12→Asn
01-1646	≥2000	Leu-120→Pro
98-2036	≥2000	Leu-159→Val
00-715	≥2000	no mutation
99-1882	1000	Pro-160→Thr
99-1914	1000	Asp-63→Glyc
02-93	1000	Val-128→Gly
02-172	1000	Ala-36→Val
98-330	1000	Thr-142→Ala
99-1916	1000	Ala-102→Val
01-172	500	Tyr-64→Asp

study, we have developed and evaluated a colorimetric method to determine the resistance to pyrazinamide that simply substitutes nicotinamide in place of pyrazinamide, thus avoiding the need for acid medium. Both pyrazinamide and nicotinamide are prodrugs, and both require the same nicotinamidase enzyme, also called PZAse, to convert them into their active acid forms: POA and nicotinic acid, respectively.[16,17] Almost all pyrazinamide resistance is associated with the loss of PZAse activity, often caused by mutations in *pncA*, the gene encoding the PZAse.[17,19,25–27] The mechanism of nicotinamide antimicrobial activity is not currently known and that of pyrazinamide is the least known among the antituberculosis drugs. It has been proposed that pyrazinamide is converted into POA inside the bacilli by nicotinamidase/pyrazinamidase and then excreted by a weak efflux pump; protonated POA is reabsorbed into the bacilli under acid conditions accumulating inside the cell and causing cellular damage.[25] A recent study has also shown that POA disrupted the membrane energetics and inhibited membrane transport function in *M. tuberculosis*.[28]

The REMA plate using nicotinamide at neutral pH, instead of pyrazinamide in acid medium, performed quite well. Compared with the Wayne and radiometric BACTEC 460-TB (300 mg/L) methods, sensitivity and specificity were, respectively, 100% and 98.6%. Susceptible strains were inhibited by 250 mg/L of nicotinamide whereas 72% of resistant strains grew well at 2000 mg/L, and 96% grew well in 1000 mg/L. We therefore propose for the REMA plate a critical concentration of 250 mg/L nicotinamide for detecting pyrazinamide resistance; strains with a nicotinamide MIC higher than 250 mg/L should be considered resistant to pyrazinamide in this test. Brander showed that on LJ medium susceptible strains were inhibited by 500 mg/L of nicotinamide,

whereas resistant strains grew well at 2000 mg/L.[14] Two isolates showed borderline resistance, with a nicotinamide MIC of 500 mg/L. One was determined to be resistant while the other was susceptible to pyrazinamide by the Wayne and BACTEC 460 (300 mg/L pyrazinamide) methods. We suggest that strains with a nicotinamide MIC of 500 mg/L in the REMA assay be tentatively labelled resistant, and that they be tested for pyrazinamide resistance using the BACTEC (300 mg/L) or Wayne methods. The REMA assay was also tested with pyrazinamide in acid media, but the bacteria grew poorly, resulting in incomplete reduction of the resazurin and erroneous results (data not shown).

Except for these two intermediate strains, the results of the REMA nicotinamide assay were identical to those obtained with the BACTEC radiometric method when using a concentration of 300 mg/L pyrazinamide, as proposed by Heifets *et al.*[6] However, when the BACTEC system was used with a pyrazinamide concentration of 100 mg/L, eight strains were falsely determined to be resistant. While it is well known that the inoculum size is critical for accurate results with the BACTEC 460-TB system it is doubtful that this was the reason for the false resistance, and, more likely, that some susceptible strains simply have pyrazinamide MICs between 100 and 300 mg/L. The radiometric BACTEC 460-TB method is commonly used for pyrazinamide DST in developed countries or laboratories having adequate resources, while the Wayne PZAse detection method is used in limited-resource countries. The newer BACTEC MGIT 960 pyrazinamide susceptibility test could replace the radiometric method, but it is still expensive for low-resource countries.[29] The Wayne method is generally reliable, but the pale change of colour can lead to difficulties in interpreting the results. A new agar medium has been recently developed and could be an attractive alternative method for pyrazinamide DST.[30]

Another method proposed for determining pyrazinamide resistance is the rapid sequencing of the *pncA* gene to detect mutations that inactivate the PZAse enzyme. There are three potential problems with this approach. First, DNA amplification and sequencing requires expensive equipment and trained personnel and remains fairly costly. Second, because of the diversity of mutations that have been associated with pyrazinamide resistance, accurate sequencing of the entire *pncA* gene is essential.[31–33] Third, as pointed out by Davies *et al.*[31] finding *pncA* mutations is not a very sensitive method for detecting pyrazinamide resistance in *M. tuberculosis*; studies have found *pncA* mutations in only 72–97% of pyrazinamide-resistant clinical isolates.[17,34] In the work reported here, mutations in the *pncA* gene could not be found in 4/22 (18%) of the pyrazinamide-resistant, PZAse-negative strains tested. Thus other mechanisms, such as POA efflux pumps, are likely involved in the development of pyrazinamide resistance.

The REMA plate assay described here, using resistance to nicotinamide as a surrogate for resistance to pyrazinamide, is a rapid and inexpensive method that could reduce the time needed to identify pyrazinamide-resistant strains. Compared with the other rapid methods, this assay costs less, requires no sophisticated equipment or special media, and could be performed routinely as part of a REMA assay for resistance to several antituberculosis drugs.[21] As the REMA assay is performed using liquid medium, biosafety considerations dictate that it can be recommended only for laboratories that have adequate facilities for *M. tuberculosis* culturing and DST,

which, however, exist in many reference laboratories in low-resource countries. Our study raises the possibility that the use of nicotinamide in antituberculosis therapy, which was abandoned in 1961, deserves to be reconsidered, especially in light of recently proposed regimens in which isoniazid is replaced by a fluoroquinolone.[35]

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Transparency declarations

None to declare.

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