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Nitrate reductase assay for the rapid detection of pyrazinamide resistance in *Mycobacterium tuberculosis* using nicotinamide

Anandi Martin[1,2]*, Andrés Cubillos-Ruiz[3], Andrea Von Groll[4], Patricia Del Portillo[3], Françoise Portaels[1] and Juan Carlos Palomino[1]

[1]Mycobacteriology Unit, Institute of Tropical Medicine, Nationalestraat 155, Antwerp 2000, Belgium; [2]Médecins Sans Frontières, rue Saint Sabin, 8, 75011 Paris, France; [3]Corpogen, Carrera 5, 66a-34, Bogota DC, Colombia; [4]Fundacion Universidade Federal do Rio Grande, Rua General Osorio, 96200-400 Rio Grande, Brazil

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Objectives: **The purpose of this study was to develop the nitrate reductase assay (NRA) for the rapid detection of pyrazinamide resistance in *Mycobacterium tuberculosis* using nicotinamide resistance as a marker of pyrazinamide resistance in Löwenstein–Jensen (LJ) medium at neutral pH.**

Methods: **We tested 68 *M. tuberculosis* isolates using nicotinamide at three different concentrations (1000, 500 and 250 mg/L) by the NRA in LJ medium and compared the results with those obtained with the BACTEC 460-TB or the BACTEC MGIT 960 as reference standard methods. Mutations in the *pncA* gene were detected by DNA sequencing of the pyrazinamide-resistant isolates.**

Results: **Out of 34 *M. tuberculosis* pyrazinamide-resistant isolates, 31 were found to be resistant to 1000 and 500 mg/L nicotinamide giving sensitivity and specificity of 91% and 94%, respectively. At 250 mg/L nicotinamide, the sensitivity and specificity decreased to 91% and 71%, respectively. Results were obtained in an average of 10 days. Based on these results, a tentative breakpoint concentration of 500 mg/L nicotinamide was defined. DNA sequencing of the *pncA* gene detected mutations in 26 out of 34 *M. tuberculosis* isolates resistant to pyrazinamide.**

Conclusions: **The NRA using nicotinamide to detect resistance to pyrazinamide in LJ medium is a rapid and accurate method that could be useful in limited-resource countries where the BACTEC 460-TB or the BACTEC MGIT 960 system is not available.**

Keywords: drug susceptibility testing, tuberculosis, *pncA*, diagnostic

Introduction

Tuberculosis (TB) kills ~2 million people each year. The global epidemic is becoming more dangerous mainly due to the presence of HIV/AIDS especially in developing countries, the emergence of multidrug-resistant TB (MDR-TB) and the recently described extensively drug-resistant TB.[1–3] Timely detection of drug resistance in patients with TB is a priority and reliable methods for rapid drug susceptibility testing (DST) are needed for better control of the disease. Pyrazinamide is an important first-line antituberculosis drug used in combination with rifampicin, isoniazid and ethambutol in the short-course TB therapy recommended by the WHO[4] and used also to treat MDR-TB. The reference method, the BACTEC TB-460, used to detect resistance of clinical isolates of *Mycobacterium tuberculosis* to pyrazinamide is difficult to perform due to the requirement of an acid pH in the medium where the growth of *M. tuberculosis* is often inhibited.[5–8] For this reason many laboratories do not perform DST for this drug. Pyrazinamide is a nicotinamide analogue prodrug that is converted to the active bactericidal form pyrazinoic acid by pyrazinamidase (PZase). Owing to structural similarities, both nicotinamide and pyrazinamide are converted by the same enzyme nicotinamidase also called pyrazinamidase to their acid forms, pyrazinoic acid (POA) and nicotinic acid, respectively. It has been shown that strains of *M. tuberculosis* resistant to pyrazinamide are also resistant to nicotinamide.[9–11]

In a previous study, we developed and evaluated a colorimetric method, the resazurin assay, to determine resistance to pyrazinamide using nicotinamide avoiding acidification of

*Corresponding author. Tel: +32-3-2476334; Fax: +32-3-2476333; E-mail: amartin@itg.be

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the medium.[12,13] Among the new rapid DST methods, the nitrate reductase assay (NRA) has been previously reported as a useful tool for the rapid and accurate detection of resistance to first-line antituberculosis drugs and it was also evaluated in a multicentre study with good results.[14] The objective of this study was to apply the NRA to detect resistance to pyrazinamide using nicotinamide at neutral pH. We compared the results with those obtained with the reference standard methods the BACTEC TB-460 or the BACTEC MGIT 960, which has now replaced the radiometric system in our laboratory. For that reason, some strains have been tested by the BACTEC TB-460 and other by the BACTEC MGIT 960 systems, and we also detected mutations in the *pncA* gene of the pyrazinamide-resistant isolates.

Materials and methods

M. tuberculosis isolates

A total of 68 *M. tuberculosis* clinical isolates were selected from the mycobacteria collection of the Institute of Tropical Medicine, Antwerp, Belgium. This panel included one-third susceptible isolates and 46 MDR-TB. These isolates have been selected to be at least resistant to isoniazid and rifampicin and originated from countries with a high prevalence of MDR-TB; in this case they were selected from Eastern European countries (Armenia, Georgia, Kazakstan, Russia and Azerbaijan). Out of 46 MDR-TB isolates, 34 were found to be pyrazinamide-resistant. Strain H37Rv susceptible to pyrazinamide (ATCC 27294) and the pyrazinamide-resistant strain (ATCC 35828) were used as controls.

Nitrate reductase assay

The NRA was performed as previously described[14] with minor modifications. Briefly, LJ medium was prepared containing potassium nitrate at 1 mg/mL and nicotinamide was included at 1000, 500 or 250 mg/L. The inoculum was adjusted to the turbidity of a no. 1 McFarland standard and diluted 1:10 in PBS, pH 7.3. For each strain, 200 μL of the undiluted inoculum was added into the nicotinamide-containing tube and 200 μL of the 1:10 dilution into two tubes without nicotinamide as growth controls. After 10 days of incubation at 37°C, 500 μL of a reagent mixture consisting of one part of 50% concentrated hydrochloric acid (HCl), two parts of 0.2% sulfanilamide and two parts of 0.1% *n*-1-naphthylethylenediamine dihydrochloride was added to one control tube. If any colour appeared, all tubes were developed with the reagent mixture; otherwise, the tubes were re-incubated and the procedure repeated at day 14. An isolate was considered resistant if colour developed in the nicotinamide-containing tube (pink to red or purple) that was darker or of the same intensity as the colour appearing in the growth control.

Susceptibility to pyrazinamide

Susceptibility to pyrazinamide was tested by the BACTEC 460-TB method or by the BACTEC MGIT 960.

BACTEC 460-TB. Susceptibility to pyrazinamide by the BACTEC 460-TB system was performed according to the manufacturer's instructions (Becton Dickinson, Sparks, MD, USA). Two vials with BACTEC pyrazinamide test medium, pH 6.0, supplemented with polyoxyethylene stearate were inoculated with 0.1 mL of bacterial suspension prepared as follows: a fresh culture on LJ was subcultured in a BACTEC 12 vial and used to inoculate one vial containing pyrazinamide at 300 mg/L and the other vial without drug used as control. The vials were incubated at 37°C and tested daily with the BACTEC 460-TB instrument. 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; and 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.

BACTEC MGIT 960. Susceptibility to pyrazinamide was tested by the BACTEC Mycobacteria Growth Indicator Tube (MGIT 960) method, according to the manufacturer's instructions (Becton Dickinson) using a freshly positive MGIT tube diluted 1:5 as inoculum. The MGIT 960 medium is a modified Middlebrook 7H9 broth with a reduced pH of 5.9. The recommended critical concentration of 100 mg/L pyrazinamide was used to discriminate between pyrazinamide-susceptible and pyrazinamide-resistant isolates. Five hundred microlitres of the inoculum was added to the pyrazinamide-containing tube and a 1:10 dilution of the inoculum was added into another tube without pyrazinamide as the growth control.

Sequencing the pncA gene

For the amplification of the pyrazinamidase/nicotinamidase gene *pncA*, the primers *pncAr* (5′-AGTCGCCCGAACGTATGGTG) and *pncAf* (5′-CCGCCGCCAACAGTTCATC) were designed on the flanking regions of the Rv2043c in order to obtain the whole coding sequence. The PCR was performed in a final volume of 50 μL containing 0.5 U of Platinum® Pfx DNA Polymerase (Invitrogen), 1× Pfx amplification buffer, 1 mM $MgSO_4$, 0.4 μM each primer, 0.3 mM dNTPs and 2 μL of DNA from culture lysate extracts. The reaction was carried out in the iCycler DNA thermal cycler (Bio-Rad) for 30 cycles consisting of 30 s of denaturation at 94°C, 30 s of annealing at 64°C and 45 s of extension at 68°C. The PCR products were verified by 1.5% agarose gel electrophoresis for the presence of a single band of 622 bp. Both strands of the *pncA* gene were sequenced using the BigDye™ terminator cycling conditions (Macrogen, Korea). The sequences were analysed using Phred, Phrap and Consed.[15–17] A sequence was considered to contain a mutation when both forward and reverse sequences showed consistent changes in high-quality bases with disagreements with the consensus sequence. The coding sequence of the *pncA* gene was translated into amino acids and aligned using ClustalW[18] and the mutations identified in the protein sequence were visualized on Bioedit 7.0.5.[19]

Results

Pyrazinamide results were obtained between 4 and 8 days with the BACTEC 460-TB and the BACTEC MGIT 960 systems and in 10 days with the nicotinamide NRA method. Results obtained by all methods were read blindly and compared later at the end of the study.

A total of 68 *M. tuberculosis* isolates have been tested by the reference methods; 34 isolates were found to be resistant to pyrazinamide and 34 were found to be susceptible. Out of the 34 pyrazinamide-resistant isolates, 31 were found to be resistant by the NRA using a concentration of 1000 mg/L nicotinamide and 3 were found to be susceptible. Out of the 34 susceptible

Table 1. Comparison of the results of nicotinamide resistance by the NRA at three different concentrations with the results of pyrazinamide by the reference methods BACTEC 460 TB/MGIT 960 for the 68 isolates of *M. tuberculosis*

	Breakpoint concentrations of nicotinamide (mg/L) used in NRA					
	1000		500		250	
Reference methods	R	S	R	S	R	S
Pyrazinamide-resistant ($n = 34$)	31	3	31	3	31	3
Pyrazinamide-susceptible ($n = 34$)	2	32	2	32	10	24
Sensitivity (%)	91		91		91	
Specificity (%)	94		94		71	

isolates, 32 were found to be susceptible by the NRA using a concentration of 1000 mg/L nicotinamide and 2 were found to be resistant (Table 1). The same results were obtained using the NRA with nicotinamide at 500 mg/L giving sensitivity and specificity of 91% and 94%, respectively. We tested the discordant isolates again and found the same results. Importantly, one isolate was found to be borderline resistant with 13% resistance in the BACTEC 460-TB system. When using a concentration of 250 mg/L nicotinamide, out of 34 pyrazinamide-resistant isolates, 31 were found to be resistant and 3 were found to be susceptible. For the 34 pyrazinamide-susceptible isolates, 24 were found to be susceptible to nicotinamide by the NRA method and 10 were found to be resistant giving sensitivity and specificity of 91% and 71%, respectively. Based on these results, a tentative breakpoint concentration of 500 mg/L nicotinamide was defined for the NRA method.

The *pncA* gene from 34 pyrazinamide-resistant isolates was sequenced to determine possible mutations in the coding region. Among the isolates analysed (Table 2), 24 had mutations within the coding sequence that consisted of nucleotide substitutions resulting in missense mutations, 2 isolates had an insertion resulting in a frameshift, isolate 72 had an insertion of one G and isolate 108 of two C nucleotides. Seven isolates showed no changes in the sequence compared with the wild-type strain. The sequence of isolate number 102 could not be determined because no amplification was obtained under every condition tested. Overall, 19 different types of mutations were found along the *pncA* gene and no isolates contained more than one mutation.

Discussion

Under routine working conditions, DST of *M. tuberculosis* to pyrazinamide is difficult to perform due to the acid pH requirement to test this drug. It has been shown that strains of *M. tuberculosis* resistant to pyrazinamide were also resistant to nicotinamide.[9] In a previous study, we demonstrated that the colorimetric resazurin assay (REMA) using nicotinamide at neutral pH instead of pyrazinamide in acid medium performed quite well.[12] In the present study, we have developed and evaluated the NRA for detecting pyrazinamide resistance using nicotinamide at neutral pH. Three concentrations of nicotinamide were tested and compared with results obtained with the reference standard methods. Most pyrazinamide-susceptible isolates were inhibited by 1000 and 500 mg/L nicotinamide. More discordant results were obtained using 250 mg/L. Therefore, a critical concentration of 500 mg/L nicotinamide is proposed to test for pyrazinamide resistance by the NRA in LJ medium. Isolates that grow at 500 mg/L nicotinamide will be considered to be resistant to pyrazinamide. This is in accordance with the results of Brander.[20] who showed in 1972 that *M. tuberculosis* was inhibited on LJ containing 500 mg/L nicotinamide. However, at this concentration our study found three isolates resistant to pyrazinamide by BACTEC MGIT 960 but susceptible by the NRA with an MIC $\leq$250 mg/L nicotinamide. Two of the three isolates, numbers 110 and 114 in Table 2, did not show any mutation in the *pncA* gene, whereas isolate 108 showed a mutation caused by the insertion of two C nucleotides that resulted in a frameshift in the final part of the C-terminal end of the pyrazinamidase protein. Our attention has been aimed to the BACTEC MGIT 960 results since it is well known that this system is very sensitive to a non-homogenous inoculum in the liquid medium. The inoculation procedure is a quite important step to avoid false-resistant results by collecting large clumps of bacteria and discrepant results might be due to MGIT inoculum density.[21,22] In our previous study,[12] we have tested pyrazinamide susceptibility of *M. tuberculosis* isolates using the BACTEC 460-TB at two concentrations of pyrazinamide; 100 mg/L as the manufacturer-recommended concentration and 300 mg/L as proposed by Heifets.[7] Our results showed that using 100 mg/L pyrazinamide, we have false-resistant results compared with the Wayne method. Results of the REMA nicotinamide test were identical to those obtained with the BACTEC TB-460 system at 300 mg/L pyrazinamide. For this reason, we also used in this study 300 mg/L pyrazinamide with the BACTEC TB-460 system. For the BACTEC MGIT 960, we followed the recommended concentration of 100 mg/L pyrazinamide. Previous reports[23–25] suggested the use of 300 mg/L pyrazinamide in the BACTEC TB-460 and in the BACTEC MGIT 960 systems for a better correlation with the reference method and the presence of *pncA* mutations. Further studies are needed to determine the best breakpoint concentration for the BACTEC MGIT 960.

The BACTEC MGIT 960 is now commonly used for DST of pyrazinamide in developed countries or laboratories having adequate resources. The Wayne method used in the past to assess pyrazinamide resistance is generally reliable, but the pale change of colour can lead to difficulties in interpretation of results. In the present study, the mutations found in the *pncA* gene were varied and scattered along the gene and showed a high diversity confirming the absence of a common hot-spot region for mutation. In general, mutations in the *pncA* gene have a good correlation with pyrazinamide resistance,[10] although resistant strains with normal PZase activity and wild-type *pncA* gene have also been reported, which indicates that alternative mechanisms of pyrazinamide resistance exist besides the lack of PZase activity.[26,27] In this study, mutations in the *pncA* gene could not be found in 8 out of 34 pyrazinamide-resistant isolates suggesting that another mechanism might be involved in conferring pyrazinamide resistance in these isolates, such as POA efflux pumps that are likely involved in the development of pyrazinamide resistance. Despite the small sample size included in

Table 2. Mutations within the *pncA* gene of the 34 pyrazinamide-resistant clinical isolates of *M. tuberculosis*

Isolate number	Change in nucleotide sequence	Change in amino acid sequence	Reference methods	Nicotinamide NRA MIC (mg/L)
2	11, (T→G)	Leu-4→Trp	BACTEC TB-460	>1000
113	11, (T→C)	Leu-4→Ser	BACTEC MGIT 960	>1000
101	28, (A→C)	Gln-10→Pro	BACTEC MGIT 960	>1000
28	34, (G→A)	Asp-12→Asn	BACTEC TB-460	>1000
8	188, (A→G)	Asp-63→Gly	BACTEC TB-460	>1000
49	190, (T→G)	Tyr-64→Asp	BACTEC TB-460	>1000
106	211, (C→A)	His-71→Asn	BACTEC MGIT 960	>1000
69	226, (A→C)	Thr-76→pro	BACTEC TB-460	>1000
72	234, insertion (G)	frameshift	BACTEC TB-460	>1000
50	254, (T→G)	Leu-85→Arg	BACTEC TB-460	>1000
105	289, (G→A)	Gly-97→Ser	BACTEC MGIT 960	>1000
117	289, (G→A)	Gly-97→Ser	BACTEC MGIT 960	>1000
34	355, (T→A)	Trp-119→Arg	BACTEC TB-460	>1000
71	355, (T→A)	Trp-119→Arg	BACTEC TB-460	>1000
7	359, (T→C)	Leu-120→Pro	BACTEC TB-460	>1000
107	389, (T→G)	Val-130→Gly	BACTEC MGIT 960	>1000
109	389, (T→G)	Val-130→Gly	BACTEC MGIT 960	>1000
11	416, (T→G)	Val-139→Gly	BACTEC TB-460	>1000
36	416, (T→G)	Val-139→Gly	BACTEC TB-460	>1000
115	424, (A→G)	Thr-142→Ala	BACTEC MGIT 960	>1000
90	424, (A→G)	Thr-142→Ala	BACTEC TB-460	>1000
116	437, (C→A)	Ala-146→Glu	BACTEC MGIT 960	>1000
111	437, (C→A)	Ala-146→Glu	BACTEC MGIT 960	>1000
104	460, (A→G)	Arg-154→Gly	BACTEC MGIT 960	>1000
53	475, (C→G)	Leu-159→Val	BACTEC TB-460	>1000
108	505, insertion (CC)	frameshift	BACTEC MGIT 960	**≤250**
110	no	no	BACTEC MGIT 960	**≤250**
114	no	no	BACTEC MGIT 960	**≤250**
32	no	no	BACTEC TB-460	>1000
64	no	no	BACTEC TB-460	>1000
73	no	no	BACTEC TB-460	>1000
103	no	no	BACTEC MGIT 960	>1000
112	no	no	BACTEC MGIT 960	>1000
102	not determined	not determined	BACTEC MGIT 960	>1000

this study, the NRA using nicotinamide as a marker of pyrazinamide resistance was shown to be a rapid and accurate method that could be useful in limited-resource countries where expensive tools are not always available. Pyrazinamide is used in the short-course therapy for TB and also in re-treatment regimens. It is important to test for pyrazinamide resistance since not all MDR-TB strains are always pyrazinamide-resistant. Out of 46 MDR-TB patients, we found 12 that were not resistant to pyrazinamide. These patients could still receive pyrazinamide in combination with other antituberculosis drugs.

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Transparency declarations

None to declare.

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