

Contributions to the diagnosis of pyrazinamide resistance in *Mycobacterium tuberculosis*

✉ Ernesto Montoro¹, Dihadenys Lemus¹, Niuris Mirabal¹, Miguel Echemendía¹, Mariela Madruga¹, Yoslane Milian¹, Raúl Díaz¹, Howard Takiff², Anandi Martin³, Patrick Van der Stuyf⁴, Juan C Palomino³

¹Laboratorio Nacional de Referencia e Investigaciones en Tuberculosis y Micobacterias Centro Colaborador OPS/OMS en Tuberculosis, Instituto de Medicina Tropical Pedro Kourí, IPK Autopista Novia del Mediodía Km 6½, AP 601, Marianao 13, La Habana, Cuba

²Centro de Microbiología y Biología Celular, CMBC Instituto Venezolano de Investigaciones Científicas, IVIC Km 11 Carretera Panamericana, Caracas, 1020A, Venezuela

³Mycobacteriology Unit, Institute of Tropical Medicine Nationalestraat, 155, 2000, Antwerp, Belgium

⁴Epidemiology and Disease Control Unit, Department of Public Health Institute of Tropical Medicine Nationalestraat, 155, 2000, Antwerp, Belgium
E-mail: emontoro@ipk.sld.cu

REPORT

ABSTRACT

The direct detection of pyrazinamide resistance in *Mycobacterium tuberculosis* is not routinely performed in many laboratories in the world because the drug is active only at acid pH, which also affects the growth of *Mycobacterium tuberculosis*. The pyrazinamidase enzyme, encoded by *pncA* gene, is necessary to convert prodrug pyrazinamide to its active form. Taking into account that nicotinamide, a structural analogue of pyrazinamide, converted in its active forms by the pyrazinamidase enzyme at a physiological pH does not affect bacterial growth, the aim of this research was to evaluate two colorimetric methods: Nitrate reductase and malachite green microtube assays, using nicotinamide to perform susceptibility testing in 102 *Mycobacterium tuberculosis* strains. The results were compared with those obtained by the classic Wayne assay. Mutations in the *pncA* gene were identified by sequencing the *pncA* gene from all isolates in which pyrazinamide resistance was detected by any of the three methods. Both the nitrate reductase and malachite green microtube assays showed sensitivities of 93.75% and specificities of 97.67%. Mutations in the *pncA* gene were found in 14 of 16 strains (87.5%) that were pyrazinamide resistant.

Keywords: tuberculosis, resistance, pyrazinamide

Biotecnología Aplicada 2011;28:265-267

RESUMEN

Aportes al diagnóstico de resistencia a pirazinamida en *Mycobacterium tuberculosis*. Los estudios de susceptibilidad a PZA no se realizan de rutina debido a que el pH ácido requerido para lograr la actividad de la droga, afecta el crecimiento de *Mycobacterium tuberculosis*. La PZA es convertida en su forma activa por acción de la enzima pirazinamidasa (PZAasa) que es codificada por el gen *pncA*. Tomando en consideración que la enzima PZAasa es la responsable de convertir la nicotinamida (NIC), droga análoga de la PZA, en su forma activa sin requerir de un pH ácido, nos propusimos evaluar dos métodos colorimétricos: Método de la Nitrato Reductasa (MNR) y Microensayo Verde Malaquita (MVM) para conocer la susceptibilidad de 102 cepas de *Mycobacterium tuberculosis* a la PZA empleando la NIC. Los resultados obtenidos se compararon con el Ensayo Enzimático de Wayne (método de referencia). A las cepas resistentes y las que mostraron resultados discordantes, se les secuenció el gen *pncA*. Se alcanzó una sensibilidad del 93.75% y una especificidad del 97.67% para el MNR y el MVM. El 87.5% de las cepas resistentes (14/16) mostraron mutaciones en el gen *pncA*.

Palabras clave: tuberculosis, resistencia, pirazinamida

Introduction

Pyrazinamide (PZA) is one of the most effect front-line drugs used in the short-course chemotherapy of tuberculosis (TB) and also in retreatment regimens for multidrug-resistant TB patients [1]. The direct detection of pyrazinamide resistance in *Mycobacterium tuberculosis* is not routinely performed in many laboratories in the world because the drug is active only at acid pH (5.5), which also affects the growth of *M. tuberculosis* [2]. For this reason, many laboratories do not perform PZA susceptibility testing, and therefore the true extent of global PZA resistance is largely unknown. The radiometric Bactec 460 system (Becton

Dickinson, Sparks, MD), using a special acid liquid medium, has been considered the reference method for detection of PZA resistance, but it requires the use of costly and problematic radioisotopes [3]. Other commercial tests, such as the nonradiometric Bactec MGIT 960 method (Becton Dickinson, Sparks, MD), utilize protocols adapted for PZA susceptibility testing, but they are also expensive and impractical for routine use in resource-poor settings [4].

M. tuberculosis pyrazinamidase (PZase) enzyme is necessary to activate prodrug PZA and most PZA-resistant strains have mutations in *pncA* gene, the

1. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity *in vitro* against semidormant *Mycobacterium tuberculosis* bacterial populations. *Am Rev Respir Dis*. 1992;145(5):1223-5.

2. Heifets L. Susceptibility testing of *Mycobacterium tuberculosis* to PZA. *J Med Microbiol*. 2002;51(1):11-2.

3. Martin A, Takiff H, Vandamme P, Swings J, Palomino JC, Portaels F. A new rapid and simple colorimetric method to detect pyrazinamide resistance in *Mycobacterium tuberculosis* using nicotinamide. *J Antimicrob Chemother*. 2006;58(2):327-31.

gene encoding this enzyme, that result in the loss or reduction of PZase activity. Thus, an indirect approach for detecting resistance is to assess PZase activity [5]. The classic way to detect PZase activity is by means of the Wayne assay [6], which monitors the hydrolysis of PZA to the active acid form, pyrazinoic acid (POA), through the color change of a ferrous ammonium phosphate solution added to the medium. Nicotinamide (NIC), a structural analogue of PZA with some activity against *M. tuberculosis*, is also converted to its active acid form, nicotinic acid, by the *M. tuberculosis* PZase [7]. Strains of *M. tuberculosis* are not only resistant to PZA but also resistant to NIC [3, 8], and the conversion of NIC into nicotinic acid by PZase occurs at a physiological pH that does not hinder bacterial growth.

In recent studies, PZA resistance was rapidly and accurately detected with the inexpensive resazurin microtiter assay (REMA) and nitrate reductase assay (NRA), using NIC as a surrogate for PZA to avoid the need for acidification of the medium [3, 8]. Another alternative colorimetric method, reported by Farnia et al., uses malachite green to test for susceptibility to first- and second line anti-TB drugs [9]. Malachite green is a triphenylmethane dye with a dark green color that becomes colorless during *M. tuberculosis* metabolism [10]. In this study, we evaluated the use of NIC in the NRA and malachite green microtube (MGMT) assay for the detection of PZA resistance and compared the results with those obtained by the Wayne assay, which served as a gold standard. We also sequenced the *pncA* gene from strains determined to be resistant by any of the three methods.

A total of 102 *M. tuberculosis* strains were tested by these methods and the results were compared with those obtained by the classic Wayne assay. Mutations in the *pncA* gene were identified by sequencing the *pncA* gene from all isolates in which pyrazinamide resistance was detected by any of the three methods. Mutations in the *pncA* gene were identified by comparison with the wild-type *M. tuberculosis pncA* gene sequence using BLAST (www.ncbi.nlm.nih.gov) or MacVector 10. All identified mutations were seen in the sequences of both strands. Statistical analysis was made using the MedCalc (Mariakerke, Belgium) software program to calculate the sensitivity (ability to detect true resistance) and the specificity (ability to detect true susceptibility).

Results and discussion

The NRA and MGMT assay using NIC were compared on the Wayne assay for the rapid detection of resistance to PZA in 102 *M. tuberculosis* strains. With the Wayne assay, the results from 78% of the strains (79 strains) were obtained at 7 days and 100% of the strains were obtained after 10 days, while the NRA method brought about the following results: 90% (91 strains) were available at day 10 and 100% at day 14. With the MGMT method, only 27% of results were available at day 10, but 100% were available at day 14. Out of 86 strains determined to be PZA susceptible by Wayne assay, 84 were susceptible and 2 resistant (strains 17 and 18) by the NRA. Similarly, with the MGMT assay 84 of these 86 strains were susceptible and 2 resistant, but the 2 discordant strains (strains 19

and 20) were not the same strains found to be discordantly resistant with the NRA (Table).

Among the 16 strains determined to be resistant to 100 mg/liter PZA with the Wayne assay, 15 were resistant in both the NRA and the MGMT assay using NIC at 500 mg/liter and 250 mg/liter, respectively, and one strain (no. 16) was susceptible in both alternative assays. From 86 strains determined to be PZA susceptible with the Wayne assay, 84 were susceptible with both the NRA and the MGMT assay. Based on these results, the sensitivity and specificity of both the NRA and the MGMT assay were 93.75% and 97.67%, respectively. The agreement between both methods and the Wayne assay was 97.05%. All strains with discordant results were retested with the NRA and MGMT assay, but the repeat susceptibility results were unchanged. The NRA results were similar to those obtained in the study by Martin et al. [8], in which the NRA, using a critical concentration of 500 mg/liter NIC, had sensitivity and specificity of 91% and 94%, respectively. Surprisingly, the sensitivity of the MGMT assay using NIC was superior to that reported by Farnia et al. (75%) [9], perhaps due to insufficient bacterial growth on the acidified medium required when PZA is used.

The Wayne assay is inexpensive and generally reliable, but the presence of a pink band in conventional Dubos agar, which indicates PZase activity and thus sensitivity to PZA, can be difficult to judge for some strains, because the band can be very faint [3, 10]. To make the assay easier to interpret and identify strains with low PZase activity, Singh et al. [10] recently recommended that for strains that are negative at 4 and 7 days, the incubation should be extended to 10 days. Our results support this longer incubation, as we found that 23 of the strains tested required 10 days to show PZase activity. If the final reading had been at day 7, these strains would have been falsely reported as resistant, and the sensitivities of the NRA and MGMT assay would have appeared to be much lower.

The results were confirmed by sequencing the *pncA* gene from the 16 strains found to be PZA resis-

4. Krüner A, Yates MD, Drobniowski FA. Evaluation of MGIT 960-based antimicrobial testing and determination of critical concentrations of first- and second-line antimicrobial drugs with drug-resistant clinical strains of *Mycobacterium tuberculosis*. *J Clin Microbiol*. 2006;44(3):811-8.

5. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. *Tuber Lung Dis*. 1997;78(2):117-22.

6. Wayne LG. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. *Am Rev Respir Dis*. 1974; 109(1):147-51.

7. McKenzie D, Malone L, Kushner S. The effect of nicotinic acid amide on experimental tuberculosis of white mice. *J Lab Clin Med*. 1948;33(10):1249-53.

8. Martin A, Cubillos-Ruiz A, Von Groll A, Del Portillo P, Portaels F, Palomino JC. Nitrate reductase assay for the rapid detection of pyrazinamide resistance in *Mycobacterium tuberculosis* using nicotinamide. *J Antimicrob Chemother*. 2008; 61(1):123-7.

9. Farnia P, Masjedi MR, Mohammadi F, Tabarsei P, Farnia P, Mohammadzadeh AR, et al. Colorimetric detection of multidrug-resistant or extensively drug-resistant tuberculosis by use of malachite green indicator dye. *J Clin Microbiol*. 2008; 46(2):796-9.

10. Jones JJ, Falkinham JO 3rd. Decolorization of malachite green and crystal violet by waterborne pathogenic mycobacteria. *Antimicrob Agents Chemother*. 2003;47(7):2323-6.

Table. Results of sequencing of the *pncA* gene of 16 *M. tuberculosis* strains that were PZA resistant by the Wayne assay and 4 strains that were resistant by one of the other two methods tested (NRA and MGMT)

Strains	Resistance/Susceptibility			<i>pncA</i> mutation
	Wayne Assay	NRA	MGMT	
1	R	R	R	L172 → P
2	R	R	R	F94 → C
3	R	R	R	Y103 → Stop codon
4	R	R	R	V155 → G
5	R	R	R	L172 → P
6	R	R	R	L172 → P
7	R	R	R	Q10 → R
8	R	R	R	VDV128-130 Del
9	R	R	R	Del nt 12 → frame shift at aa. 4
10	R	R	R	V139 → A
11	R	R	R	V139 → A
12	R	R	R	T76 → P
13	R	R	R	no mutation
14	R	R	R	no mutation
15	R	R	R	W119 → Stop codon
16	R	S	S	K48 → T
17	S	R	S	T135 → P
18	S	R	S	no mutation
19	S	S	R	no mutation
20	S	S	R	no mutation

tant with the Wayne assay, as well as the 4 strains determined to be resistant only with the NRA or MGMT technique. Of the 16 strains that were PZA resistant by the Wayne assay, 14 (87.5%) had mutations in the *pncA* gene, confirming that this is the principal mechanism for loss of PZase activity *M. tuberculosis* [11]. Surprisingly, the two strains (13 and 14) without mutations in the *pncA* gene or the 65 bases upstream were found to be PZA resistant in all the three methods. It is possible that these strains, for unknown reasons, have very low PZase activity that is below the limits of detection for the three methods [10]. Alternatively, they could have mutations in another site, such as a transporter for PZA, or the target of POA. Strain 16 is curious because it was resistant by the Wayne assay and has a K48T *pncA* mutation, but it showed PZase activity on both the NRA and MGMT methods. One possible explanation could be that these two methods are more sensitive at detecting low PZase activity than the Wayne assay. However, this would not explain why each of these alternative methods failed to detect PZase activity (indicating PZA resistance) in two different strains where the Wayne assay detected activity (indicating PZA sensitivity). This notion of increased sensitivity is also inconsistent with the finding that strain 17 was PZA sensitive on the Wayne assay but resistant with the NRA contained a T135P *pncA* mutation. If the amino acid substitution had only a minimal effect on the activity of PZase protein, in this case the NRA was less sensitive in detecting the remaining activity. Further studies are needed to evaluate the structure-function significance of particular amino acid substitutions. The remaining three strains with false resistant results (strains 18, 19, and 20), *i.e.*, strains that were PZA sensitive by the Wayne assay but resistant with one of the other assays, showed no mutations in their *pncA* coding regions or in the 65 bases upstream of the first codon.

The mutations found in our PZA-resistant strains were distributed throughout the *pncA* gene, confirming the high diversity and absence of mutation hot spots reported in other studies, but the L172P substitution was present in three strains and the V139A substitution in two. Perhaps these were multiple isolates of the same strain from different patients. Also, similar to findings in previous studies, the majority of the *pncA* mutations in our PZA-resistant strains (12/13, 92.3%) were single-base-pair substitutions, although some deletions were also found [3, 12]. It should be pointed out that these three assays will detect only strains that are resistant because they lack of the PZase activity required to convert PZA to its active form, POA, and will not detect the few strains that are resistant due to mutations in other sites, such as in the drug target for POA [13].

The costs of the NRA and MGMT assay have been evaluated in different studies. In Norway, Syre *et al.*

[14] have estimated the price of the NRA as \$3.00 per isolate for two drugs, compared with \$21.00 for the Bactec 460 method and \$23.00 for the manual mycobacterial growth indicator tube (MGIT). In Argentina, Mengatto *et al.* [15] compared the costs to test one isolate for two drugs and estimated \$19.52 for the manual MGIT and \$0.17 for the NRA [21]. Recently, Farnia *et al.* [9] reported for the MGMT assay a cost of \$2.50, using 12 drugs per strain. Additional studies are required to establish the cost-effectiveness of the NRA and MGMT assay compared with the conventional methods to demonstrate the benefit of these technologies. Studies to evaluate the costs of these two methods and their direct application with sputum samples are in progress in our laboratory.

Relevance

This study is the first international scientific report that evaluates and demonstrates the high reliability of two colorimetric methods using nicotinamide for pyrazinamide resistance detection in *M. tuberculosis*. It is also the first time that the Tuberculosis National Reference Laboratory of Cuba has obtained results about the sequence of one gene associated to *M. tuberculosis* drug resistance. This has confirmed the worth of the two alternative methods mentioned above and also has contributed to the knowledge of *M. tuberculosis* drug resistance. NRA and MGMT for PZA susceptibility testing could be implemented in low-resource countries due to their simplicity because they do not require the use of sophisticated equipment. The availability of reliable methods to detect resistance to pyrazinamide will allow us to know level of resistance to this drug. Consequently, a proper antibiotic treatment regimen could be implemented in patients with resistant strains. Finally, a reduction of the community transmission of the resistant pathogen could be accomplished.

The knowledge and experience obtained in the field of TB resistance situates our institution as reference laboratory not only at a national level but also international one since these methods could be implemented in other countries of the region, where TB and MDR constitute a major health problem.

Conclusions

Determination of PZA resistance by both the NRA and MGMT methods, using NIC as a surrogate for PZA, showed a high level of agreement with the Wayne assay. The MGMT assay should be easy to implement in clinical TB laboratories, and its microtube format may pose fewer biosafety risks than colorimetric microplate methods. Particularly, the NRA and MGMT assay with NIC are simple, rapid, accurate, inexpensive, and robust alternatives for PZA susceptibility testing, not requiring sophisticated equipment and able to be implemented in low-resource countries.

11. Huang TS, Lee SS, Tu HZ, Huang WK, Chen YS, Huang CK, *et al.* Correlation between pyrazinamide activity and *pncA* mutations in *Mycobacterium tuberculosis* isolates in Taiwan. *Antimicrob Agents Chemother.* 2003;47(11):3672-3.

12. Juréen P, Werngren J, Toro JC, Hoffner S. Pyrazinamide resistance and *pncA* gene mutations in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother.* 2008; 52(5):1852-4.

13. Zimhony O, Vilchèze C, Arai M, Welch JT, Jacobs WR Jr. Pyrazinoic acid and its n-propyl ester inhibit fatty acid synthase type I in replicating tubercle bacilli. *Antimicrob Agents Chemother.* 2007;51(2):752-4.

14. Syre H, Phyu S, Sandven P, Bjorvatn B, Grewal HM. Rapid colorimetric method for testing susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampin in liquid cultures. *J Clin Microbiol.* 2003;41(11):5173-7.

15. Mengatto L, Chiani Y, Imaz MS. Evaluation of rapid alternative methods for drug susceptibility testing in clinical isolates of *Mycobacterium tuberculosis*. *Mem Inst Oswaldo Cruz.* 2006;101(5):535-42.