

## Evaluation of Colorimetric Methods Using Nicotinamide for Rapid Detection of Pyrazinamide Resistance in *Mycobacterium tuberculosis*<sup>∇</sup>

Niuris C. Mirabal,<sup>1</sup> Sergio L. Yzquierdo,<sup>1</sup> Dihadenys Lemus,<sup>1</sup> Mariela Madruga,<sup>1</sup> Yoslane Milián,<sup>1</sup> Miguel Echemendía,<sup>1</sup> Howard Takiff,<sup>2</sup> Anandi Martin,<sup>3</sup> Patrick Van der Stuyf,<sup>4</sup> Juan Carlos Palomino,<sup>3</sup> and Ernesto Montoro<sup>1\*</sup>

Laboratorio Nacional de Referencia en Tuberculosis y Mycobacteria, PAHO/WHO Collaborating Centre, Instituto de Medicina Tropical “Pedro Kouri,” Havana, Cuba<sup>1</sup>; Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela<sup>2</sup>; Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp, Belgium<sup>3</sup>; and Epidemiology and Disease Control Unit, Department of Public Health, Institute of Tropical Medicine, Antwerp, Belgium<sup>4</sup>

Received 16 February 2010/Returned for modification 7 May 2010/Accepted 4 June 2010

**The direct detection of pyrazinamide resistance in *Mycobacterium tuberculosis* is sufficiently difficult that many laboratories do not attempt it. Most pyrazinamide resistance is caused by mutations that inactivate the pyrazinamidase enzyme needed to convert the prodrug pyrazinamide to its active form. We evaluated two newer and simpler methods to assess pyrazinamidase activity, the nitrate reductase and malachite green microtube assays, using nicotinamide in place of pyrazinamide. A total of 102 strains were tested by these methods and the results compared with those obtained by the classic Wayne assay. Mutations in the *pncA* gene were identified by sequencing the *pncA* genes 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 that were pyrazinamide resistant and in 1 of 4 strains that were sensitive by the Wayne assay. Both of these simple methods, used with nicotinamide, are promising and inexpensive alternatives for the rapid detection of pyrazinamide resistance in limited-resource countries.**

Due to its activity against semidormant bacilli sequestered within macrophages, pyrazinamide (PZA) is one of the most effect frontline drugs used in the short-course chemotherapy of tuberculosis (TB) and also in retreatment regimens for multi-drug-resistant TB patients (6, 36). However, the detection of PZA resistance is difficult and often unreliable because the drug is active only at acid pH (5.5), which also affects the growth of *Mycobacterium tuberculosis* (5, 37). 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 (20, 30). Other commercial tests, such 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 (14, 27).

PZA is a prodrug that requires activation by the *M. tuberculosis* pyrazinamidase (PZase), and most PZA-resistant strains have mutations in *pncA*, the gene encoding this enzyme, that result in the loss or reduction of PZase activity (7). Thus, an indirect approach to detecting resistance is to assess PZase

activity (22). The classic way to detect PZase activity is with the Wayne assay (12, 35), 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 (12, 24). Strains of *M. tuberculosis* that are resistant to PZA are also resistant to NIC (18, 20, 28), 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 (18, 20). Another alternative colorimetric method, reported by Farnia et al., uses malachite green to test for susceptibility to first- and second-line anti-TB drugs (4). Malachite green is a triphenylmethane dye with a dark green color that becomes colorless during *M. tuberculosis* metabolism (9).

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* genes from strains determined to be resistant by any of the three methods.

### MATERIALS AND METHODS

**Strain selection.** A total of 102 *M. tuberculosis* strains from the collection of the National Reference Tuberculosis Laboratory of the Instituto de Medicina

\* Corresponding author. Mailing address: Laboratorio Nacional de Referencia en Tuberculosis y Mycobacteria, PAHO/WHO Collaborating Centre, Instituto de Medicina Tropical “Pedro Kouri” (IPK), Autopista Novia del Mediodía Km 61/2, La Lisa, La Habana, Cuba. Phone: 53-7-2020448. Fax: 53-7-2046051. E-mail: EMontoro@ipk.sld.cu.

<sup>∇</sup> Published ahead of print on 16 June 2010.

Tropical "Pedro Kouri" were evaluated. *M. tuberculosis* H37Rv (ATCC 27294), which is susceptible to PZA, and *M. tuberculosis* (ATCC 35828), which is resistant to PZA, were used as controls.

**Nitrate reductase assay.** The NRA was carried out as described by Angeby et al. (1). Briefly, Löwenstein-Jensen (LJ) medium, containing potassium nitrate at 1,000 mg/liter and NIC (Sigma-Aldrich, St. Louis, MO) at 500 mg/liter, was prepared as described by Martin et al. (18). The inoculum turbidity was adjusted to a McFarland tube no. 1 standard and diluted 1:10 in phosphate-buffered saline. For each strain, 200  $\mu$ l of the undiluted suspension was inoculated into the NIC-containing tube and 200  $\mu$ l of the 1:10 dilution was inoculated into three drug-free tubes as growth controls. The tubes were incubated at 37°C. After 7 days, 500  $\mu$ l of a reagent mixture consisting of 1 part of 50% concentrated hydrochloric acid (HCl), 2 parts of 0.2% sulfanilamide, and 2 parts of 0.1% *n*-1-naphthylethylenediamine dihydrochloride was added to one control tube. If any color (strong or weak pink) was noted, all tubes were developed with the reagent mixture; otherwise, the tubes were reincubated and the procedure was repeated on days 10 and 14. A strain was considered resistant if the color that developed in the NIC-containing tube (pink to red or purple) was as dark or darker than the color appearing in the growth control tube.

**Malachite green microtube assay.** The MGMT assay was performed in 1.5-ml microtubes according to the protocol described previously by Farnia et al. (4) but with the use of a 0.05-mg/ml solution of malachite green (Sigma-Aldrich, St. Louis, MO). The concentration of NIC used in Middlebrook 7H9-S broth (Becton Dickinson, Sparks, MD) was 250 mg/liter, as described by Martin et al. (20). In brief, the turbidity of the bacterial suspension was adjusted to McFarland tube no. 1 standard and further diluted 1:5 in Middlebrook 7H9-S broth. Five microtubes were used for each strain: 200  $\mu$ l of drug-free Middlebrook 7H9-S broth was added to one microtube as a control for the medium, 100  $\mu$ l of drug-free Middlebrook 7H9-S broth was added to three microtubes used as growth controls, and 100  $\mu$ l of NIC-containing Middlebrook 7H9-S broth was added to the fifth microtube. Subsequently, 100  $\mu$ l of the inoculum diluted 1:5 was added to each microtube except that for the medium control. After 7 days of incubation at 37°C, 50  $\mu$ l of the 0.05-mg/ml solution of malachite green was added into one growth control and the medium control microtubes, and both were incubated at 37°C for 12 to 24 h. If any loss of color occurred in the medium control microtube, the medium was considered to be contaminated and the assay was repeated. If the medium control was unchanged but there was a loss of the dark green color in the growth control microtube, 50  $\mu$ l of the malachite green solution was added to a second growth control microtube and to the corresponding NIC-containing microtube, and both were incubated for another 12 to 24 h at 37°C. If no color change was seen in the growth control microtube, the remaining microtubes were reincubated and the procedure repeated on day 10 or 14. A strain was considered resistant if the green color in the NIC-containing microtube decreased or disappeared.

**Wayne assay.** PZase activity was assayed by the Wayne assay (35), which was modified to extend the time of incubation to 10 days for those strains that did not show PZase activity (no pink band seen) after 7 days of incubation. Briefly, a heavy loopful of an actively growing culture of *M. tuberculosis* in LJ medium was carefully inoculated onto the surfaces of three screw-cap tubes containing Dubos agar (Becton Dickinson, Sparks, MD) with 100 mg/liter PZA (Sigma-Aldrich, St. Louis) and 2 mg/ml sodium pyruvate. The tubes were incubated at 37°C for 4 days, after which 1 ml of ferrous ammonium phosphate solution (1%) was added to each tube. After 30 min, the tubes were examined for the appearance of a pink band (positive) in the subsurface agar. Negative tubes were refrigerated for 4 h and reexamined. If the reaction was negative after 4 h of incubation, the remaining tubes were developed at day 7 or 10. The pink color indicates the enzymatic hydrolysis of the PZA into free POA, so that a strain was considered susceptible to PZA if a pink band, indicating PZase activity, appeared in the agar. In contrast, a culture showing no pink color in the agar was considered to lack PZase activity and therefore to be resistant to PZA.

**PCR amplification and automated sequencing.** DNA was extracted as described by van Soolingen et al. (34). The *pncA* gene was amplified using the forward and reverse primers P1 (5'-GTCGGTCATGTTTCGCGATCG-3', beginning 105 bp upstream of *pncA*) and P6 (5'-GCTTTGCCGAGCGCTCCA-3', beginning 60 bp downstream of the *pncA* stop codon) described by Scropio et al. (29). 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 for 40 cycles; followed a final elongation at 72°C for 10 min. The expected size of the *pncA* PCR products was 720 bp. The PCR products were purified by using a Qiagen gel extraction kit (Qiagen, West Sussex, United Kingdom) according to the manufacturer's protocols and sequenced with an automated DNA sequencer, model ABI 3130XL (Applied Biosystems, Foster City, CA), using the same P1 and P6 primers. Mutations in the *pncA* gene were identified by comparison with the wild-type *M. tuberculosis pncA* gene sequence

TABLE 1. Time required for the determining PZA resistance or sensitivity of 102 *M. tuberculosis* strains by the Wayne assay, NRA, and MGMT assay

Test	No. of strains with the following time (days) required for result:			
	4	7	10	14
Wayne assay	54	25	23	
NRA		38	53	11
MGMT assay		2	26	74

using BLAST ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) or MacVector 10. All identified mutations were seen in the sequences of both strands.

**Statistical analysis.** The MedCalc (Mariakerke, Belgium) software program was used to calculate the sensitivity (ability to detect true resistance) and the specificity (ability to detect true susceptibility).

## RESULTS

The NRA and MGMT assay using NIC were compared to the Wayne assay for the rapid detection of resistance to PZA in 102 *M. tuberculosis* strains. With the Wayne assay, results from 78% of the strains (79 strains) were obtained by 7 days and results from 100% of the strains were obtained after 10 days, while with the NRA method, results for 90% (91 strains) were available by day 10 and for 100% by day 14. With the MGMT method, only 27% of results were available at day 10, but 100% were available by day 14 (Table 1). Out of 86 strains determined to be PZA susceptible by Wayne assay, 84 were susceptible and 2 resistant (no. 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 (no. 19 and 20) were not the same strains found to be discordantly resistant with the NRA.

Of the 16 strains determined to be resistant to 100 mg/liter PZA with the Wayne assay, 15 were resistant with 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 with both alternative assays. Of the 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.

In a second phase of the study, we sequenced the *pncA* genes from the 16 strains determined to be PZA resistant by Wayne assay, as well as the 4 strains found to be resistant by one of the other two assays (Table 2). Of the 16 PZA-resistant strains, 10 had *pncA* mutations resulting in amino acid substitutions, 2 had mutations resulting in stop codons, 1 had a deletion of three amino acids, and 1 had a nucleotide deletion causing a frameshift. In two strains, (no. 13 and 14), no *pncA* mutations were found. Among the four strains determined to be PZA sensitive by the Wayne assay but resistant with one of the other methods, only one (no. 17) had a mutation in the *pncA* coding region. This strain was found to be resistant only with the NRA. Overall, 12 different mutations were found in the *pncA*

TABLE 2. Results of sequencing of the *pncA* genes of 16 *M. tuberculosis* strains that were PZA resistant by the Wayne assay and of 4 strains that were resistant by one of the other two methods tested

Strain no.	Result <sup>a</sup> for:			<i>pncA</i> mutation
	Wayne assay	NRA	MGMT assay	
1	R	R	R	L-172 → P
2	R	R	R	F-94 → C
3	R	R	R	Y-103 → stop codon
4	R	R	R	V-155 → G
5	R	R	R	L-172 → P
6	R	R	R	L-172 → P
7	R	R	R	Q-10 → R
8	R	R	R	VDV128-130 deletion
9	R	R	R	Deletion nucleotide 12 → frameshift at amino acid 4
10	R	R	R	V-139 → A
11	R	R	R	V-139 → A
12	R	R	R	T-76 → P
13	R	R	R	None
14	R	R	R	None
15	R	R	R	W-119 → stop codon
16	R	S	S	K-48 → T
17	S	R	S	T-135 → P
18	S	R	S	None
19	S	S	R	None
20	S	S	R	None

<sup>a</sup> R, resistant; S, susceptible.

gene, with the L172P substitution present in three strains and the V139A substitution in two strains.

## DISCUSSION

As PZA is given in the early intensive phase of antituberculosis treatment (36), there is an urgent need, especially in low-resource countries, for a simple, reliable, and inexpensive method for rapidly determining whether a strain is susceptible to this drug. However, in spite of its very high *in vivo* activity, *in vitro* the susceptibility testing of *M. tuberculosis* against PZA is difficult to perform because the drug is active only at low pH (5, 37). Among the conventional methods, the radiometric Bactec TB-460 has proven to be satisfactory and has been considered the most reliable technique for PZA susceptibility testing in developed countries, while the Wayne assay can be used as an alternative where the automated culture methods are not available (13).

In the past, NIC, an analogue of PZA, was found to have antituberculosis activity (23, 24). It was reported that strains of *M. tuberculosis* that were resistant to PZA were also resistant to NIC and that PZA susceptibility could be reliably determined using high concentrations of NIC as a surrogate, thus avoiding the need for acid media (3, 11, 18, 20). Based on these reports, Martin et al. standardized the REMA plate and NRA for detecting PZA resistance at neutral pH using NIC, with critical concentrations of 250 mg/liter for Middlebrook 7H9-S broth and 500 mg/liter for LJ medium (18, 20). The MGMT assay is a new colorimetric method reported to accurately assess susceptibility to the first- and second-line antituberculosis drugs, including PZA, but there are no reports of its use

with NIC (4). In the present study, we evaluated the NRA and MGMT assay for detecting resistance to PZA using NIC at the critical concentrations previously defined (18, 20).

Compared with the Wayne assay, the NRA and MGMT techniques using NIC showed identical sensitivities and specificities of 93.75% and 97.67%, respectively. The NRA results were similar to those obtained in the study by Martin et al. (18), 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%) (4), 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 (20, 31). To make the assay easier to interpret and to identify strains with low but present PZase activity, Singh et al. (31) 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 (Table 1). 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* genes from the 16 strains found to be PZA resistant with the Wayne assay, as well as the 4 strains determined to be resistant only with the NRA or MGMT technique. Of 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* (2, 8). Surprisingly, the two strains (no. 13 and 14) without mutations in the *pncA* gene or the 65 bases upstream were found to be PZA resistant with all 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 (31). 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 with 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 one strain (no. 17) that was PZA sensitive with 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 (no. 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 (10, 15, 18), 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 (10, 18, 32). It should be pointed out that these three assays will detect only strains that are resistant because they lack the PZase activity necessary 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 (38).

The costs of the NRA and MGMT assay have been evaluated in different studies. In Norway, Syre et al. (33) 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. (25) 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. (4) 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.

In summary, 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. NRA was successfully tested in previous studies (1, 17–19) and has the advantage of being performed on classical LJ medium that does not need to be acidified. 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 (4, 16, 26). The NRA and MGMT assay with NIC are simple, rapid, accurate, inexpensive, and robust alternatives for PZA susceptibility testing that do not require sophisticated equipment and could be implemented in low-resource countries.

#### ACKNOWLEDGMENTS

This work was supported in part by the Belgian Directorate-General for Development Cooperation and by Cuba-Venezuela Project no. 689.

#### REFERENCES

- Angeby, K. A., L. Klintz, and S. E. Hoffner. 2002. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. *J. Clin. Microbiol.* **40**:553–555.
- Barco, P., R. F. Cardoso, R. D. C. Hirata, C. Q. F. Leite, J. R. Pandolfi, D. N. Sato, M. L. Shikama, F. Fiúza de Melo, E. M. Mamizuka, P. A. Z. Campanerut, and M. H. Hirata. 2006. *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* clinical isolates from the southeast region of Brazil. *J. Antimicrob. Chemother.* **58**:930–935.
- Brander, E. 1972. A simple way of detecting PZA resistance. *Tubercle* **53**:128–131.
- Farnia, P., M. Reza, F. Mohammadi, P. Tabarsei, P. Farnia, A. R. Mohamadzadeh, P. Baghei, M. Varahram, S. Hoffner, and A. A. Velayati. 2008. Colorimetric detection of multidrug-resistant or extensively drug-resistant tuberculosis by use of malachite green indicator dye. *J. Clin. Microbiol.* **46**:796–799.
- Heifets, L. 2002. Susceptibility testing of *Mycobacterium tuberculosis* to PZA. *J. Med. Microbiol.* **51**:11–12.
- Heifets, L., and P. Lindholm-Levy. 1992. Pyrazinamide sterilizing activity *in vitro* against semidormant *Mycobacterium tuberculosis* bacterial populations. *Am. Rev. Respir. Dis.* **145**:1223–1225.
- Hirano, K., M. Takahashi, Y. Kaumi, Y. Fukasawa, and C. Abe. 1997. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. *Tuber. Lung Dis.* **78**:117–122.
- Huang, T. S., S. S. J. Lee, H. Z. Tu, W. K. Huang, Y. S. Chen, C. K. Huang, S. R. Wann, H. H. Lin, and Y. C. Liu. 2003. Correlation between pyrazinamide activity and *pncA* mutations in *Mycobacterium tuberculosis* isolates in Taiwan. *Antimicrob. Agents Chemother.* **47**:3672–3673.
- Jones, J. J., and J. O. Falkinham. 2003. Decolorization of malachite green and crystal violet by waterborne pathogenic mycobacteria. *Antimicrob. Agents Chemother.* **47**:2323–2326.
- Juréen, P., J. Werngren, J. C. Toro, and S. Hoffner. 2008. Pyrazinamide resistance and *pncA* gene mutations in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **52**:1852–1854.
- Kalich, R., W. Gerloff, R. Neubert, and H. Ulber. 1978. Determination of the sensitivity of mycobacteria to pyrazinamide and nicotinamide. *Z. Erkr. Atmungsorgane.* **151**:103–112.
- Konno, K., F. M. Feldman, and W. McDermott. 1967. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. *Am. Rev. Respir. Dis.* **95**:461–469.
- Krishnamurthy, A., D. Almeida, C. Rodrigues, and A. Mehta. 2004. Comparison of pyrazinamide drug susceptibility of *Mycobacterium tuberculosis* by radiometric BACTEC and enzymatic pyrazinamidase assay. *Indian J. Med. Microbiol.* **22**:166–168.
- Kruuner, A., M. D. Yates, and F. A. Drobniowski. 2006. 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.* **44**:811–818.
- Lee, K. W., J. Lee, and K. Jung. 2001. Characterization of *pncA* mutations of pyrazinamide-resistant *Mycobacterium tuberculosis* in Korea. *J. Korean Med. Sci.* **16**:537–543.
- Lemus, D., A. Martin, E. Montoro, F. Portaels, and J. C. Palomino. 2004. Rapid alternative methods for detection of rifampicin resistance in *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* **54**:130–133.
- Lemus, D., E. Montoro, M. Echemendía, A. Martin, F. Portaels, and J. C. Palomino. 2006. Nitrate reductase assay for detection of drug resistance in *Mycobacterium tuberculosis*: simple and inexpensive method for low-resource laboratories. *J. Med. Microbiol.* **55**:861–863.
- Martin, A., A. Cubillos-Ruiz, A. Von Groll, P. Del Portillo, F. Portaels, and J. C. Palomino. 2008. Nitrate reductase assay for the rapid detection of pyrazinamide resistance in *Mycobacterium tuberculosis* using nicotinamide. *J. Antimicrob. Chemother.* **61**:123–127.
- Martin, A., E. Montoro, D. Lemus, N. Simboli, N. Morcillo, M. Velasco, J. Chauca, L. Barrera, V. Ritacco, F. Portaels, and J. C. Palomino. 2005. Multicenter evaluation of the nitrate reductase assay for drug resistance detection of *Mycobacterium tuberculosis*. *J. Microbiol. Methods* **63**:145–150.
- Martin, A., H. Takiff, P. Vandamme, J. Swings, J. C. Palomino, and F. Portaels. 2006. A new rapid and simple colorimetric method to detect pyrazinamide resistance in *Mycobacterium tuberculosis* using nicotinamide. *J. Antimicrob. Chemother.* **58**:327–331.
- Martin, A., S. Panaiotov, F. Portaels, S. Hoffner, J. C. Palomino, and K. Angeby. 2008. The nitrate reductase assay for the rapid detection of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. *J. Antimicrob. Chemother.* **62**:56–64.
- McClatchy, J. K., A. Y. Tsang, and M. S. Cernich. 1981. Use of pyrazinamidase activity on *Mycobacterium tuberculosis* as a rapid method for determination of pyrazinamide susceptibility. *Antimicrob. Agents Chemother.* **20**:556–557.
- McDermott, W., and R. Tompsett. 1954. Activation of PZA and nicotinamide in acidic environment *in vitro*. *Am. Rev. Tuberc.* **70**:748–754.
- McKenzie, D., L. Malone, and S. Kushner. 1948. The effect of nicotinic acid amide on experimental tuberculosis of white mice. *J. Lab. Clin. Med.* **33**:1249–1253.
- Mengatto, L., Y. Chiani, and M. S. Imaz. 2006. Evaluation of rapid alternative methods for drug susceptibility testing in clinical isolates of *Mycobacterium tuberculosis*. *Mem. Inst. Oswaldo Cruz* **101**:535–542.
- Montoro, E., D. Lemus, M. Echemendía, A. Martin, F. Portaels, and J. C. Palomino. 2005. Comparative evaluation of the nitrate reductase assay, the MTT test, and the resazurin microtitre assay for drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* **55**:500–505.
- Scarparo, C., P. Ricordi, G. Ruggiero, and P. Piccoli. 2004. Evaluation of the fully automated BACTEC MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide, streptomycin, isoniazid, ri-

- fampin, and ethambutol and comparison with the radiometric BACTEC 460TB method. *J. Clin. Microbiol.* **42**:1109–1114.
28. **Scorpio, A., and Y. Zhang.** 1996. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat. Med.* **2**:662–667.
  29. **Scorpio, A., P. Lindholm-Levy, L. Heifets, R. Gilman, S. Siddiqi, M. Cynamon, and Y. Zhang.** 1997. Characterization of *pncA* mutations in PZA-resistant *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **41**: 540–543.
  30. **Siddiqi, S.** 2004. BACTEC 460TB system-indirect susceptibility tests with pyrazinamide for *Mycobacterium tuberculosis*, p. 7.8.2.1–7.8.2.3. In H. D. Isenberg (ed.), *Clinical microbiology procedures handbook*, 2nd ed., vol. 2. ASM Press, Washington, DC.
  31. **Singh, P., C. Wesley, G. P. S. Jadaun, S. K. Malonia, R. Das, P. Upadhyay, J. Faujdar, P. Sharma, P. Gupta, A. K. Mishra, K. Singh, D. S. Chauhan, V. D. Sharma, U. D. Gupta, K. Venkatesan, and V. M. Katoch.** 2007. Comparative evaluation of Löwenstein-Jensen proportion method, BacT/ALERT 3D system, and enzymatic pyrazinamidase assay for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **45**:76–80.
  32. **Sreevatsan, S., X. Pan, Y. Zhang, B. N. Kreiswirth, and J. M. Musser.** 1997. Mutations associated with pyrazinamide resistance in *pncA* of *Mycobacterium tuberculosis* complex organisms. *Antimicrob. Agents Chemother.* **41**: 636–640.
  33. **Syre, H., S. Phyu, P. Sandven, B. Bjorvatn, and H. M. S. Grewal.** 2003. Rapid colorimetric method for testing susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampin in liquid cultures. *J. Clin. Microbiol.* **41**:5173–5177.
  34. **van Soolingen, D., P. W. M. Hermans, P. E. W. de Hass, D. R. Soll, and J. D. A. van Embden.** 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J. Clin. Microbiol.* **29**:2578–2586.
  35. **Wayne, L. G.** 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. *Am. Rev. Respir. Dis.* **109**:147–151.
  36. **World Health Organization.** 2008. Global tuberculosis control: surveillance, planning, financing. WHO report 2008. WHO/HTM/TB/2008.393. World Health Organization, Geneva, Switzerland.
  37. **Zhang, Y., A. Scorpio, H. Nikaido, and Z. Sun.** 1999. Role of acid pH and deficient efflux of pyrazinoic acid in unique susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *J. Bacteriol.* **181**:2044–2049.
  38. **Zimhony, O., C. Vilchèze, M. Arai, J. T. Welch, and W. R. Jacobs, Jr.** 2007. Pyrazinoic acid and its *n*-propyl ester inhibit fatty acid synthase type I in replicating tubercle bacilli. *Antimicrob. Agents Chemother.* **51**:752–754.