ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1999, p. 2317–2319 Vol. 43, No. 9
0066-4804/99/$04.00+0

Relationship between Pyrazinamide Resistance, Loss of Pyrazinamidase Activity, and Mutations in the *pncA* Locus in Multidrug-Resistant Clinical Isolates of *Mycobacterium tuberculosis*

M. MESTDAGH,[1] P. A. FONTEYNE,[2] L. REALINI,[2] R. ROSSAU,[3] G. JANNES,[3] W. MIJS,[3] K. A. L. DE SMET,[3] F. PORTAELS,[2] AND E. VAN DEN EECKHOUT[1]*

Laboratory for Pharmaceutical Biotechnology, FFW, University of Ghent, 9000 Ghent,[1] Unit of Mycobacteriology, Institute of Tropical Medicine, 2000 Antwerp,[2] and Innogenetics n.v., 9052 Ghent,[3] Belgium

Received 25 March 1999/Returned for modification 16 June 1999/Accepted 13 July 1999

Sixty-two *Mycobacterium tuberculosis* isolates were tested for pyrazinamidase activity, and their pyrazinamide susceptibility was determined by the radiometric method. Sequencing of *pncA* genes in the 23 resistant strains revealed mutations in 16 pyrazinamidase-negative strains, 11 of which had not been previously described. Six isolates containing wild-type *pncA* might possess alternative resistance mechanisms.

Pyrazinamide (PZA), one of the first-line drugs for tuberculosis treatment, is assumed to be a prodrug, converted by the bacterial enzyme pyrazinamidase (PZase) to the toxic pyrazinoic acid (POA), the target of which remains unknown (7). Since most PZA-resistant *Mycobacterium tuberculosis* strains have lost PZase activity, enzyme activity assays are sometimes used to replace PZA susceptibility testing, which is problematic because of growth problems at the acid pH (±5.5) required for in vitro activity of PZA (2, 4). Single mutations in the *pncA* gene encoding this PZase are considered the major PZA resistance mechanism in *M. tuberculosis* (5, 11, 12, 15). Our objective was to gain further insight into PZA resistance mechanisms and their molecular-biological background by comparing different methods for detection of PZA resistance in *M. tuberculosis*.

Fifty-four multidrug-resistant *M. tuberculosis* strains were isolated from clinical specimens collected in Bangladesh, Azerbaijan, Siberia, Belgium, Abkhazia, Rwanda, Congo-Brazzaville, Kazakhstan, and Canada, and eight isolates were obtained from the Scottish Mycobacteria Reference Laboratory (SMRL). *M. tuberculosis* H37Ra (NCTC 7417) was included as a PZA-susceptible control strain. One *Mycobacterium bovis* isolate from Belgium and one from Egypt from the collection of the Institute of Tropical Medicine were used as PZA-resistant control organisms. *M. tuberculosis* strains were grown to confluence on Löwenstein-Jensen medium, and *M. bovis* strains were grown on Stonebrink medium.

Isolates were tested for PZA susceptibility by the BACTEC radiometric method (3, 10, 13) at 100 μg of PZA per ml according to the manufacturer's instructions (14).

The PZase activity of all 65 isolates was assayed, following the modified version of the Wayne (17) test, as described by Jakschik (6). For each isolate, one test tube and one control tube were prepared. The test medium contained 6.5 g of Dubos broth base (Difco Laboratories, Detroit, Mich.), 1.0 g of PZA (Janssen Chimica, Geel, Belgium), 2.0 g of sodium pyruvate (Janssen Chimica), and 15.0 g of agar (Difco Laboratories) in 1 liter of water. In the control medium, PZA was omitted. Freshly prepared autoclaved media were divided into 5-ml aliquots in glass tubes and were allowed to cool in the upright position. Next, 0.2 to 0.3 ml of sterilized water was aseptically added to both test and control tubes. Tubes were inoculated with a loopful of a freshly grown culture of each isolate by stabbing the agar two to three times onto the bottom. After incubation at 37°C for 4 days, 1.0 ml of a freshly prepared 1% ferro(II) ammonia sulfate (Acros Chimica, Geel, Belgium) solution was added to each tube, and the tubes were kept at 4°C for 4 h. A PZase activity assay was considered positive when a dark red to brownish ring appeared on the agar-water interface.

Genomic DNA was extracted as described previously (16). *pncA* genes were amplified by PCR (9) with primers P1 and P6 (12) to obtain DNA fragments of about 720 bp. The cycling parameters were 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; 40 cycles were performed. These PCR products were used to perform cycle sequencing by using fluorescence-labelled dideoxynucleotide terminators with the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) and primers P1, P3, P4, and P6 (12) according to the manufacturer's instructions. Sequence data were generated with an automated DNA sequencer, model 373A (Applied Biosystems).

Thirty-nine isolates were susceptible to PZA; they all displayed PZase activity. The *pncA* sequence of these isolates was not determined, because earlier investigations (11, 12, 15) of sequences in 75 PZA-susceptible *M. tuberculosis* strains did not reveal any nucleotide change. Twenty-three isolates were resistant to PZA. For these strains, sequencing of the *pncA* open reading frame and an 85-bp upstream, putative regulatory region was performed. The results obtained are summarized in Table 1. In 16 PZA-resistant isolates, the lack of PZase activity was correlated with mutations leading to amino acid substitutions (10 of 16), large deletions (1 of 16), frameshifts (4 of 16) by single nucleotide insertion or deletion, or promoter mutations (1 of 16). In four PZA-resistant isolates lacking PZase activity, wild-type *pncA* sequence was present. Three PZA-resistant isolates displayed PZase activity: the wild-type *pncA* sequence was present in two of them, while the other contained a nucleotide change leading to an amino acid substitu-

* Corresponding author. Mailing address: Laboratory for Pharmaceutical Biotechnology, FFW, University of Ghent, Harelbekestraat 72, 9000 Ghent, Belgium. Phone: 32-9.264.80.52. Fax: 32-9.220.66.88. E-mail: Elfriede.VandenEeckhout@rug.ac.be.

TABLE 1. PZase activity, changes in *pncA* nucleotide sequence, and corresponding amino acid changes of the 23 PZA-resistant *M. tuberculosis* isolates in this study

Isolate	Origin	PZase assay result	Change(s) in:	
			Nucleotide sequence	Amino acid sequence
8251	Rwanda	−	Δ C at 512	Frameshift
94-735	Congo	+	No	No
97-56	Siberia	−	No	No
97-58	Siberia	−	No	No
97-69	Siberia	−	No	No
97-553	Belgium	−	C to G at 514 and T to C at 515	L172A
97-557	Belgium	−	C to G at 514 and T to C at 515	L172A
97-578	Bangladesh	−	C to T at 425[a]	T142M
97-718	Azerbaijan	−	T to G at 254	L85R
97-720	Azerbaijan	−	C insertion 407–408	Frameshift
97-826	Canada	−	T to C at 307	Y103H
97-854	Bangladesh	−	G to A at 511	A171T
97-983	Bangladesh	−	No	No
97-1006	Bangladesh	−	A to C at 410[a]	H137P
97-1052	Azerbaijan	+	No	No
97-1069	Azerbaijan	−	Δ 68 bp 195–263	Frameshift
97-1174	Azerbaijan	−	C to A at 185	P62H
98-29	Scotland	−	G insertion 221–222	Frameshift
98-30	Scotland	−	G insertion 221–222	Frameshift
98-31	Scotland	−	C to T at 151	H51Y
98-32	Scotland	+	C to T at 512	A171V
98-34	Scotland	−	G to C at 406	D136H
98-36	Scotland	−	A to G at −11[b]	No

[a] Previously described mutations (12).
[b] Previously described mutation (15).

tion (isolate 98-32 [Ala to Val at codon 171]). For strains with discrepant results between susceptibility testing and the PZase assay (PZA resistant, but PZase positive) or between the PZase assay and *pncA* sequencing (PZase negative, but *pncA* wild type), the results obtained were confirmed in a second test round.

Detection of PZase activity, as described by Wayne (17) and modified by Jakschik (6), is an easy and very rapid technique suited for any mycobacterial laboratory. Considering BACTEC PZA susceptibility determination as a reference, PZase negativity was correlated with PZA resistance in all 20 strains in this study. However, for only 39 of the 42 PZase-positive strains, a PZA-susceptible pattern was obtained on BACTEC. This confirms the findings of Butler and Kilburn (1) and Miller et al. (8) that PZA-resistant isolates are not always PZase negative. Our conclusion is that PZase test results should only be interpreted in one direction—a negative result indicating PZA resistance—whereas care should be taken in interpreting positive test results.

Mutations were present in 17 (74%) of the 23 PZA-resistant isolates as defined by the BACTEC radiometric method. The six strains lacking *pncA* mutations are divided into two groups by the PZase test: one group of two strains was PZase positive, and a second group of four strains was PZase negative. Resistance to PZA in the first group may be due to mutations in the POA drug target, which has not yet been found. The second group could have undergone downregulation of the *pncA* gene, directed on the translation phase or on regulation of transcription, as an alternative to inactivation of the gene product by point mutations in the open reading frame. Two of these strains had a completely identical restriction fragment length polymorphism pattern (97-56 and 97-58 [data not shown]). Additional research on these strains should help to elucidate the mechanism of action of the antituberculous agent POA.

This work was supported by a grant of the Institute for Enhancement of the Scientific-Technological Research in the Industry (IWT) of the Flemish Government and by grant no. G.0368.98 of the Funding for Scientific Research in Belgium.

Clinical specimens from Bangladesh were obtained thanks to A. Van Deun, and eight *M. tuberculosis* isolates were obtained from the Scottish Mycobacteria Reference Laboratory thanks to John Norton (Murex Biotech Limited). We thank the team of M. Van Montagu (University of Ghent) for preparation of the primers.

REFERENCES

1. **Butler, W. R., and J. O. Kilburn.** 1983. Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob. Agents Chemother. **24:**600–601.
2. **Cutler, R. R., P. Wilson, J. Villarroel, and F. V. Clarke.** 1997. Evaluating current methods for determination of the susceptibility of mycobacteria to pyrazinamide, conventional, radiometric Bactec and two methods of pyrazinamidase testing. Lett. Appl. Microbiol. **24:**127–132.
3. **Heifets, L. B., and R. C. Good.** 1994. Current laboratory methods for the diagnosis of tuberculosis, p. 85–110. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection and control. American Society for Microbiology, Washington, D.C.
4. **Hewlett, D., Jr., D. L. Horn, and C. Alfalla.** 1995. Drug-resistant tuberculosis: inconsistent results of pyrazinamide susceptibility testing. JAMA **273:**916–917.
5. **Hirano, K., M. Takahashi, Y. Kazumi, Y. Fukasawa, and C. Abe.** 1998. Mutation in pncA is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. Tuber. Lung Dis. **78:**117–122.
6. **Jakschik, M.** 1986. Reports of the Paris 30 Sept. – 1 Oct. 1985 Sessions of the IUAT Scientific Committees. Bull. Int. Union Tuberc. **61:**57–61.
7. **Konno, K., F. M. Feldmann, and W. McDermott.** 1967. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am. Rev. Respir. Dis. **95:**461–469.
8. **Miller, M. A., L. Thibert, F. Desjardins, S. H. Siddiqi, and A. Dascal.** 1995. Testing of susceptibility to pyrazinamide: comparison of Bactec method with pyrazinamidase assay. J. Clin. Microbiol. **33:**2468–2470.
9. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
10. **Salfinger, M., and L. B. Heifets.** 1988. Determination of pyrazinamide MICs for *Mycobacterium tuberculosis* at different pHs by the radiometric method.

Antimicrob. Agents Chemother. **32:**1002–1004.
11. **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.
12. **Scorpio, A., P. Lindholm-Lévy, L. Heifets, R. Gilman, S. Siddiqi, M. Cynamon, and Y. Zhang.** 1997. Characterization of the *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **41:**540–543.
13. **Siddiqi, S. H.** 1992. Radiometric (BACTEC) tests for slowly growing mycobacteria, p. 5.14.1–5.14.25. *In* H. D. Isenberg (ed.), Clinical microbiology procedures handbook, vol. 1. American Society for Microbiology, Washington, D.C.
14. **Siddiqi, S. H.** 1989. BACTEC TB system, product and procedure manual. Becton Dickinson Diagnostic Instruments Systems, Towson, Md.
15. **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.
16. **van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, 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.
17. **Wayne, L. G.** 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am. Rev. Respir. Dis. **109:**147–151.