J Korean Med Sci 2001; 16: 537-43
ISSN 1011-8934

Characterization of *pncA* Mutations of Pyrazinamide-Resistant *Mycobacterium tuberculosis* in Korea

Pyrazinamide (PZA) is one of the most important drugs for the treatment of *Mycobacterium tuberculosis* infection. However, the increasing frequency of PZA-resistant strains limits its effectiveness. In Korea, most PZA-resistant strains also exhibit both isoniazid and rifampin resistance making it essential to identify these resistant strains accurately and rapidly for effective treatment of mycobacterial infection. In this study, the characteristics and frequency of mutations of the *pnc*A gene encoding pyrazinamidase were investigated in PZA-resistant clinical isolates from Korea. Automated DNA sequencing was used to evaluate the usefulness of DNA-based detection of PZA resistance. Among 95 PZA-resistant clinical isolates, 92 (97%) exhibited mutations potentially affecting either the production or the activity of the enzyme. Mutations were found throughout the *pnc*A gene including the upstream region. Single nucleotide replacement appeared to be the major mutational event (69/92), although multiple substitutions as well as insertion and deletion of nucleotides were also identified. The high frequency of *pnc*A mutations observed in this study supports the usefulness of DNA-based detection of PZA-resistant *M. tuberculosis*. Having verified the scattered and diverse mutational characteristics of the *pnc*A gene, automated DNA sequencing seems to be the best strategy for rapid detection of PZA-resistant *M. tuberculosis*.

Key Words : *Mycobacterium tuberculosis; Drug Resistance; Pyrazinamide, pncA; Mutation*

Kyung Wha Lee*, Jae-Myung Lee†, Ki-Suck Jung†

Department of Clinical Pathology*, and Division of Pulmonary Medicine†, College of Medicine, Hallym University, Seoul, Korea

Received : 4 May 2001
Accepted : 21 June 2001

Address for correspondence
Kyung Wha Lee, Ph.D.
Department of Clinical Pathology, Kangdong Sacred Heart Hospital, Hallym University,
445 Gil-dong, Kangdong-ku, Seoul 134-701, Korea
Tel : +82.2-2224-2327, Fax : +82.2-6413-6119
E-mail : leekw@hallym.or.kr

INTRODUCTION

Tuberculosis still remains one of the leading causes of morbidity and mortality worldwide. Controlling tuberculosis becomes more difficult by the emergence of drug-resistant *Mycobacterium tuberculosis*. The global surveillance program reported that resistance to anti-tuberculous drugs was found in all 35 countries and has become a global problem (1). The overall prevalence was 12.6% for single drug resistance (range, 2.3-42.4%) and 2.2% for multidrug resistance (range, 0-22.1%). Patients infected with drug-resistant *M. tuberculosis* strains are less likely to be cured, particularly if they are suffering from recurrent infections, malnourished, or infected with human immunodeficiency virus (HIV) (2). Second-line antituberculous drugs for patients infected with drug resistant *M. tuberculosis* are usually not bactericidal and more expensive (3).

According to a Korean national survey of 2,486 *M. tuberculosis* strains isolated from patients without prior treatment, 282 (11.3%) isolates exhibited resistance to one or more drugs (4). Among the 282 drug resistant strains, 19 (6.7%) were resistant to pyrazinamide (PZA). Interestingly, all PZA resistant strains showed resistance to other first-line drugs such as isoniazid, rifampin, and ethambutol. With this background, an accurate and rapid method to determine PZA susceptibility is crucial for the successful treatment of multidrug resistant tuberculosis in Korea.

PZA is a prodrug that is converted to bactericidal pyrazinoic acid by pyrazinamidase (PZase) produced by *M. tuberculosis*. Functioning exclusively in the acid environment (pH 5.0-5.5) at the site of bacterial infection (5), PZA can kill semi-dormant (6) and probably intracellular *M. tuberculosis* (7). This characteristic of PZA makes it possible to reduce total duration of treatment from 12-18 months to 6 months (8, 9).

PZA susceptibility is usually determined by growth of *M. tuberculosis* on a medium containing PZA by measuring the minimum inhibitory concentration (MIC) (10). Alternatively, PZA susceptibility has also been determined by detecting PZase activity of the cultured *M. tuberculosis* (11, 12) as PZase activity is lost in PZA-resistant isolates (13). However, these conventional culture-based methods require up to 3 months and exhibit high discordance rates among laboratories (14). A major source of discordance seems to

originate from the low pH environment of the medium that is required for conversion of PZA to pyrazinoic acid, but is undesirable for growth of *M. tuberculosis* (15).

In 1996, the gene in *M. tuberculosis* encoding PZase, *pncA*, was characterized (16). The gene consists of 561 bp encoding a 20 kDa enzyme with 186 amino acids. Mutation of the *pncA* gene was suggested as the major mechanism of PZA resistance. Several subsequent studies including 20-40 PZA-resistant *M. tuberculosis* isolates from different countries confirmed the close correlation between *pncA* mutation and PZA resistance (72-97%) (17-23). These observations raised the possibility of utilizing DNA-based methods as an alternative PZA susceptibility test.

Since *M. tuberculosis* isolates from each country exhibit a unique DNA- fingerprint (24), it is possible that the genetic factors controlling resistance may vary. In addition, recent reports have suggested that mutations in the *pncA* gene could be specific to subtypes of *M. tuberculosis* (23) and/or to the geographic source of the isolate (17). In this study, the *pncA* gene sequence was characterized in 95 multidrug resistant, PZase negative *M. tuberculosis* strains isolated from Korean patients to define any possible geographic preferences for mutations in the gene, and to evaluate the utility of a DNA-based detection test for PZA-resistant *M. tuberculosis* isolates in Korea.

MATERIALS AND METHODS

Bacterial strains

Two strains of *M. tuberculosis*, H37Rv (ATCC 27294) and *M. tuberculosis* (ATCC 35806), were included as PZA-susceptible controls. Ninety-five PZA-resistant *M. tuberculosis* isolates from sputum cultures grown on Ogawa medium were obtained from the Korean Institute of Tuberculosis, which participated in the WHO-IUATLD global project (1). Resistance to PZA was determined by the PZase test using the Wayne method (25). All PZA-resistant strains showed concomitant resistance to isoniazid, rifampin, and ethambutol.

pncA gene amplification

Bacterial DNA was extracted using a kit according to the manufacturer's instructions (DNA PrepMate-M, Bioneer Corp, Cheongwon, Korea). The *pncA* gene was amplified from each *M. tuberculosis* isolate using primers, P1 and P6, as previously described by Scorpio et al. (22). These primers anneal -105 bp upstream of the start codon and 55 bp downstream of the stop codon of the *pncA* gene, respectively. The expected size of the PCR product was 720 bp, which included the full length of the *pncA* gene (561 bp). An additional PCR primer set, P0 (annealing -177 bp upstream of the *pncA* gene) and P2 (annealing at nucleotide positions 91 to 110 bp of the *pncA* gene), was used for several selected isolates to study a larger segment of the upstream region in the gene. The size of the PCR product using this second PCR primer set was 287 bp in length.

PCR amplifications were carried out in a GeneAmp PCR system 9600 thermocycler (Perkin-Elmer Corp., Foster City, CA, U.S.A.). Reaction mixtures (100 μL) contained 0.1-0.5 μg bacterial DNA, 50 pmol of each PCR primer, 200 μmol of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 2.5 units *Taq* DNA polymerase (Bioline, London, U.K.), 15 mM ammonium sulfate, 50 mM Tris-HCl (pH 8.8), 50 μM EDTA, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 10 mM beta-mercaptoethanol, and 10% (v/v) DMSO. The reaction mixtures were subjected to 5 min at 96℃ followed by 35 cycles of 20 sec at 96℃, 30 sec at 55℃, 60 sec at 72℃ and terminated by an additional 10 min at 72℃. Successful gene amplifications were confirmed by UV transillumination following electrophoresis on a 1% agarose gel stained with ethidium bromide in 1 × TAE buffer.

DNA sequencing analysis

PCR products from each *M. tuberculosis* strain were purified with the PCR purification kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions. The purified PCR product was subjected to a sequencing reaction using a BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (PE-Applied Biosystems, CA, U.S.A.) and primers. The PCR primers were used in the sequencing reaction to obtain complete sequence information from both strands of the gene. Reaction products were analyzed on a 5% Long Ranger gel (FMC BioProducts, Rockland, Maine, U.S.A.) using an ABI Prism 377 DNA Sequencer (PE-Applied Biosystems). Sequences were analyzed using Sequence Navigator software (PE-Applied Biosystems).

RESULTS

The majority, 92 of the 95 *M. tuberculosis* clinical isolates (97%), exhibited mutations in the *pncA* gene as compared to the wild type (PZA sensitive) sequence (Table 1). These included 73 variations at 71 dispersed sites throughout the *pncA* gene (Fig. 1A). Thirteen variations were found in more than two isolates and 44 mutations were newly identified in this study. The size of the alteration varied from one to 80 bp, although most of the mutations involved only a single nucleotide (81/92; 88%). In contrast, no mutations were found in the two PZA-susceptible *M. tuberculosis* strains. The three PZA-resistant isolates that did not contain any alterations and the two PZA-susceptible strains, were subjected to further sequence analysis of the upstream regulatory region

Table 1. *pncA* mutations identified in PZA-resistant *M. tuberculosis* isolates* in Korea

Changes		No. of isolates
Nucleotide substitution (position†)	Amino acid substitution (residue)	
A→G (-11)	None	6
G→T (3)‡	Met→Ile (1)	1
T→G (11)‡	Leu→Trp (4)	1
G→T (19)	Val→Phe (7)	1
T→A (20)‡	Val→Asp (7)	1
A→G (23)‡	Asp→Gly (8)	1
T→G (26)‡	Val→Gly (9)	1
T→C (26)‡	Val→Ala (9)	1
C→T (28)	Gln→stop (10)	1
A→C (29)	Gln→Pro (10)	3
A→G (29)	Gln→Arg (10)	1
A→C (29), T→G (295)‡	Gln→Pro (10), Tyr→Asp (99)	1
A→C (35)	Asp→Ala (12)	1
T→C (40)	Cys→Arg (14)	1
G→A (41)‡	Cys→Tyr (14)	1
GT→TC (68-69)‡	Gly→Val (23)	1
CC→AA (74-75), C→G (77), C→A (83)‡	Ala→Glu (25), Ala→Gly (26), Ala→Asp (28)	1
T→G (134)‡	Val→Gly (45)	1
C→A (137)	Ala→Glu (46)	1
A→G (139)	Thr→Ala (47)	1
A→C (146)‡	Asp→Ala (49)	1
C→G (169)	His→Asp (57)	1
T→C (172)	Phe→Leu (58)	1
T→G (190)‡	Tyr→Asp (64)	1
T→C (199)	Ser→Pro (67)	1
T→C (202)‡	Trp→Arg (68)	2
T→G (202)‡	Trp→Gly (68)	1
G→A (203)	Trp→stop (68)	2
G→C (203)‡	Trp→Ser (68)	1
A→C (226)	Thr→Pro (76)	2
C→T (227)	Thr→Ile (76)	1
G→A (233)‡	Gly→Asp (78)	1
T→G (254)	Leu→Arg (85)	1
T→C (254)	Leu→Pro (85)	1
A→C (286)‡	Lys→Gln (96)	1
A→G (286)	Lys→Glu (96)	3
T→C (307)	Tyr→His (103)	2
A→C (308)‡	Tyr→Ser (103)	2
C→G (309)	Tyr→stop (103)	1
G→A (357)‡	Trp→stop (119)	1
G→C (362)	Arg→Pro (121)	1
A→C (403)‡	Thr→Pro (135)	2
G→A (406)‡	Asp→Asn (136)	2
G→T (406)‡	Asp→Tyr (136)	1
A→C (410)	His→Pro (137)	1
G→A (415)	Val→Met (139)	3
C→T (421)‡	Gln→stop (141)	1
A→C (422)	Gln→Pro (141)	2
G→A (436)‡	Ala→Thr (146)	1
T→G (464)	Val→Gly (155)	1
T→G (476)‡	Leu→Arg (159)	1
T→C (515)	Leu→Pro (172)	1
G→T (538)‡	Val→Phe (180)	1
G deletion (77)‡		1
80 nucleotides deletion (151-230)‡		1
A deletion (158)‡		1
C deletion (161)‡		1
TACAG deletion (307-311)‡		1
C deletion (341)		1
GG deletion (381-382)‡		1
ATGT deletion (386-389)‡		1
G deletion (406)‡		1
T deletion (452)‡		1
T insertion (287)‡		1
18 nucleotide insertion (368)‡		1
AG insertion (382)‡		1
GG insertion (391)		1
G insertion (392)‡		1
G insertion (414)‡		1
T insertion (465)‡		2
TGAC insertion (480)‡		1
C insertion (532)‡		1

*, All isolates lacked PZase activity and showed multiple resistance to isoniazid, rifampin, and ethambutol. †, Number of nucleotide position was counted from the start codon (ATG) of *pncA* gene. Nucleotides in the 5′-upstream region of the gene are indicated by minus numbers. ‡, Newly identified mutations in this study

(up to -177), but no alterations from the wild type sequence were found.

Mutations included nucleotide substitutions (72/92 isolates), deletions (10/92), and insertions (10/92) of the *pncA* gene (Table 2). Three different types of nucleotide substitutions were observed. First, six isolates carried mutations at -11 (A→G), the upstream region of the gene. This mutation was the most frequent alteration observed in this study. Secondly, six isolates created a stop codon by single nucleotide substitutions at positions 28, 203, 309, 357, or 421, presumably leading to premature termination of protein synthesis (Table 1). The remaining 60 isolates exhibited

Table 2. Characteristics of *pncA* mutations in PZA-resistant *M. tuberculosis* isolates in Korea

Type of mutation	Frequency (%)	Size of mutation
Nucleotide substitution	72/92 (78%)	
Upstream region (-11)	6/72	1 bp
Creates stop codon	6/72	1 bp
Amino acid substitution	60/72	1-4 bp
Nucleotide deletion*	10/92 (11%)	1-80 bp
Nucleotide insertion*	10/92 (11%)	1-18 bp

*Results of mutation cause a frame shift presumably resulting in either an abnormal or a prematurely truncated polypeptide

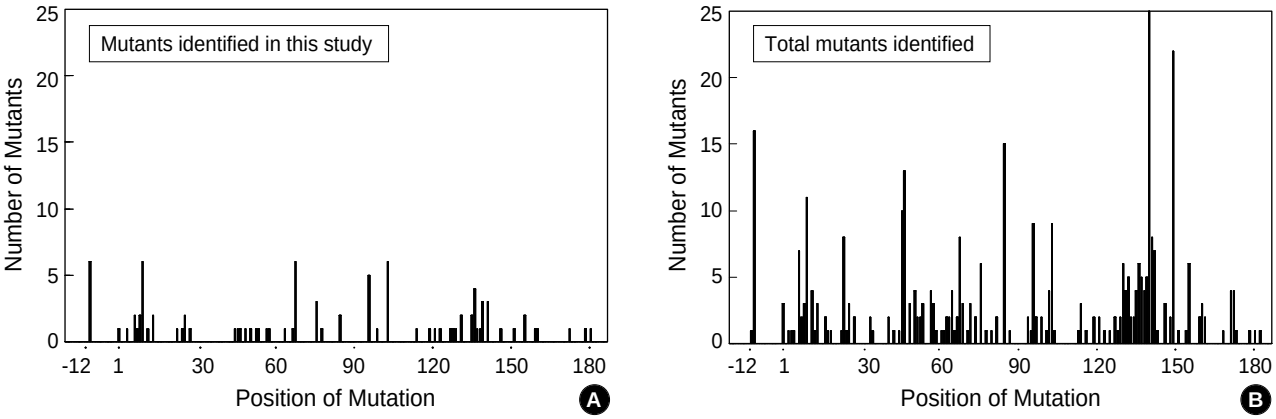

Fig. 1. Locations of *pncA* mutations found in PZA-resistant *M. tuberculosis*. Mutations in the 5′-upstream region of the gene are indicated as nucleotide position and labeled by minus numbers. Mutations in the coding region are labeled by positive codon numbers. The gene is 186 codons in length. Mutations have been found from -12 (T→G) through the entire coding region of the gene (20). (A) Location of alterations in 92 mutants identified in this study, (B) Location of alterations obtained from 254 previously reported mutants (16-23, 26, 37) as well as from the 92 mutants in this study.

nucleotide substitutions resulting in amino acid replacements. All alterations affecting the coding sequence appear to alter the protein sequence or expression; none was synonymous (silent).

A unique mutation at position 169 (CAC→GAC), defined originally in *Mycobacterium bovis*, was also identified in one *M. tuberculosis* isolate (16). Three isolates carried substitutions at more than two sites (Table 1). One isolate carried nucleotide substitutions at positions 29 (A to C) and 295 (T to G) resulting in amino acid changes Gln→Pro (residue 10) and Tyr→Asp (residue 99), respectively. The second isolate carried two nucleotide substitutions at positions 68 (G to T) and 69 (T to C) resulting in a single amino acid change Gly→Val at residue 23. The third isolate carried four nucleotide substitutions at positions 74 (C to A), 75 (C to A), 77 (C to G), and 83 (C to A) resulting in Ala→Glu (residue 25), Ala→Gly (residue 26), and Ala→Asp (residue 28), respectively.

Nucleotide deletions were found in 10 isolates and ranged from one to 80 bp (Table 1). In four isolates, more than two nucleotides were deleted. Nucleotide insertions were also found in 10 isolates and all of them occurred in the 3′-half of the gene. Nucleotide insertions ranged from one to 18 bp. In four isolates, more than two nucleotides were inserted. Deletion or insertion of nucleotides caused reading frame shift presumably resulting in either an abnormal or a prematurely truncated polypeptide.

DISCUSSION

The relationship between PZA resistance and mutation of the *pncA* gene has been under study ever since the gene was identified and characterized (16). More than 200 PZA-susceptible *M. tuberculosis* strains failed to show any mutations of the gene including silent substitutions (18-20, 23). In contrast, diverse mutations at various sites of the *pncA* gene were identified in PZA-resistant strains and the frequencies of mutation varied among studies (75-100%) (17-23). In this study of Korean isolates, the frequency of *pncA* mutation appeared to be very high (97%) in PZA-resistant *M. tuberculosis*. This suggests that a DNA-based testing method detecting mutation in the *pncA* gene would be very useful as an alternative PZA susceptibility test in Korea. The method would overcome several problems encountered in conventional methods such as limitations in sensitivity, reliability, and the time requirements of the assay.

The DNA-based method, however, would still fail to detect some PZA-resistant isolates, for example, three PZA-resistant isolates with defective PZase activity in this study which did not exhibit any mutations in the *pncA* gene. These *M. tuberculosis* isolates have been observed previously (17, 21). In addition, PZA-resistant isolates retaining PZase activity, which are expected to carry a normal *pncA* gene, have also been reported (21, 26, 27). This implies that other mechanisms are involved in PZA resistance. Recently, two alternative mechanisms have been proposed: active efflux of bactericidal pyrazinoic acid from the organism (28) and defects in PZA uptake of the organism (29, 30). Defects in other genes, such as those required for PZase expression, might also be a source of PZA resistance. Based on reports described thus far, the frequency of *M. tuberculosis* in this category seems to be fairly low and the detection of these isolates should rely on conventional culture methods until the exact mechanisms are defined. Thus, initial screening by DNA-based method followed by conventional culture-based method for any mutation negative isolates, might be appropriate.

Several characteristics of *pncA* gene mutation in *M. tuberculosis* can be pointed out based on the data obtained in this and previously reported studies. First of all, the mutations

of the gene were too diverse to address any geographic preference. The 73 types of variation including 44 never observed previously, were identified from 92 mutants in this study (Table 1). Other studies also reported new variants. Secondly, the types of mutation are diverse: nucleotide substitutions (78%), deletions (11%), and insertions (11%) have all been observed. This characteristic was also observed in previous studies (19, 21-23). There appears to be a higher tendency toward nucleotide deletion and insertion in the *pncA* gene compared to mutations found in *rpoB* gene related to rifampin-resistance (31).

In addition, most of the mutations involved a single nucleotide (81/92, 88%) and majority of them was nucleotide substitution (69/81). Every mutation found thus far seems to be detrimental to the function of the PZase since all isolates were selected based on a defect in enzyme activity. This implies that the function of the enzyme is very sensitive to sequence alterations in any region of the polypeptide. In general, single amino acid substitutions have been known to be sufficient to eradicate or reduce stability of the enzyme by changes in conformation and/or alteration of hydrogen bonds or salt bridges of the polypeptide (32, 33). Interestingly, 25% of the replacement of amino acid (15/60) involved proline. Proline has been well known to affect the conformation of the polypeptide (34).

One of the mutants identified in this study carried a single nucleotide substitution at codon 57 (CAC→GAC) resulting in an amino acid substitution (His→Asp) (Table 1). This is a unique mutation specific to *M. bovis*, which is naturally resistant to PZA (16). This difference has been used to discriminate *M. tuberculosis* from *M. bovis* (35, 36). Since others have also reported PZA-resistant *M. tuberculosis* mutants carrying the same mutation (23), special caution should be taken in the discrimination of the two organisms using only the polymorphism at codon 57 of the *pncA* gene. Instead of using it alone, several other polymorphisms in other genes, such as *oxyR*, unique to either *M. tuberculosis* or *M. bovis*, should be tested simultaneously (35).

Lastly, the mutations were dispersed throughout the *pncA* gene including the upstream region (Fig. 1). This scattered pattern of mutation is more obvious when all reported data are combined (Fig. 1B). To date, mutations have been found to alter 44% of the codons (81/186 codons) with no specific hot spots of variation. It should be noted that the high frequencies of mutation at some codons (e.g., codons 140 and 149) were the result of the spread of single mutant strain during any outbreak (17). This is very unusual compared to other drug-related genes, such as *rpoB* and *katG*, where several restricted sites are involved in the conversion to drug resistance (31, 38). This implies that the whole region encompassing the *pncA* may be a hot spot of mutation in the genome.

The most frequent mutation in this study (6/92, 6.5%) involved nucleotide -11, 5′-of the coding region, with a single nucleotide substitution (A→G). The same mutant was also identified in other studies (2, 17, 21, 23) with the highest frequency of this mutation reported in Russia (5/31, 16%) (20). It is well known that this region upstream of the start codon (AUG) of each mRNA is a critical site for the correct positioning of the ribosome in the initiation of protein synthesis (39). Thus, this substitution may prevent appropriate binding of the ribosome to the *pncA* mRNA leading to a failure in PZase protein synthesis. This hypothesis must be confirmed by further study.

Conclusively, this study confirmed the usefulness of DNA-based detection of PZA-resistant *M. tuberculosis*. Considering the dispersed and diverse mutational characteristics of the *pncA* gene, an automated DNA sequencing approach appears to be the best strategy for rapid detection of PZA-resistant *M. tuberculosis*. In addition, the high diversity of the *pncA* mutations will be epidemiologically useful in tracing the outbreak or transmission of PZA-resistant *M. tuberculosis* strains (17, 40, 41).

ACKNOWLEDGMENTS

This research was supported by a grant from the Hallym University Medical Center Research Fund (#01-2000-13). We thank Miss JS Lee for technical assistance.

REFERENCES

1. Pablos-Mendez A, Raviglione MC, Laszlo A, Binkin N, Rieder HL, Bustreo F, Cohn DL, Lambregts-van Weezenbeek CS, Kim SJ, Chaulet P, Nunn P. *Global surveillance for antituberculosis-drug resistance, 1994-1997. N Engl J Med 1998; 338: 1641-9.*
2. Frieden TR, Sherman LF, Maw KL, Fujiwara PI, Crawford JT, Nivin B, Sharp V, Hewlett D, Brudney K, Alland D, Kreisworth BN. *A multi-institutional outbreak of highly drug-resistant tuberculosis: epidemiology and clinical outcomes. JAMA 1996; 276: 1229-35.*
3. Mahmoudi A, Iseman MD. *Pitfalls in the care of patients with tuberculosis. Common errors and their association with the acquisition of drug resistance. JAMA 1993; 270: 65-8.*
4. Kim SJ, Bai GH, Hong YP. *Drug-resistant tuberculosis in Korea, 1994. Int J Tuber Lung Dis 1997; 1: 302-8.*
5. McDermott W, Tompsett R. *Activation of pyrazinamide and nicotinamide in acidic environment in vitro. Am Rev Tuberc 1954; 70: 748-54.*
6. Heifets L, Lindholm-Levy P. *Pyrazinamide sterilizing activity in vitro against semidormant Mycobacterium tuberculosis bacterial populations. Am Rev Respir Dis 1992; 145: 1223-5.*
7. Mitchison DA. *The action of antituberculosis drugs in short-course chemotherapy. Tubercle 1985; 66: 219-25.*
8. Snider DE Jr, Rogowski J, Zierski M, Bek E, Long MW. *Successful intermittent treatment of smear-positive pulmonary tuberculosis in*

six months: a cooperative study in Poland. Am Rev Respir Dis 1982; 125: 265-7.

9. Steele MA, Des Prez RM. *The role of pyrazinamide in tuberculosis chemotherapy. Chest 1988; 94: 845-50.*
10. Salfinger M, Heifets LB. *Determination of pyrazinamide MICs for Mycobacterium tuberculosis at different pH by the radiometric method. Antimicrob Agents Chemother 1988; 32: 1002-4.*
11. McClatchy JK, Tsang AY, Cernich MS. *Use of pyrazinamidase activity on Mycobacterium tuberculosis as a rapid method for determination of pyrazinamide susceptibility. Antimicrob Agents Chemother 1981; 20: 556-7.*
12. Trivedi SS, Desai SG. *Pyrazinamidase activity of Mycobacterium tuberculosis-a test of sensitivity to pyrazinamide. Tubercle 1987; 68: 221-4.*
13. Konno K, Feldmann FM, McDermott W. *Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am Rev Respir Dis 1967; 95: 461-9.*
14. Hewlett D, Horn DL, Alfalla C. *Drug-resistant tuberculosis: inconsistent results of pyrazinamide susceptibility testing. JAMA 1995; 273: 916-7.*
15. Stottmeier KD, Beam RE, Kubica GP. *Determination of drug susceptibility of mycobacteria to pyrazinamide in 7H10 agar. Am Rev Respir Dis 1967; 96: 1072-5.*
16. Scorpio A, Zhang Y. *Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med 1996; 2: 662-7.*
17. Cheng S, Thibert L, Sanchez T, Heifets L, Zhang Y. *pncA mutations as a major mechanism of pyrazinamide resistance in Mycobacterium tuberculosis: spread of a monoresistant strain in Quebec, Canada. Antimicrob Agents Chemother 2000; 44: 528-32.*
18. 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: 117-22.*
19. Lemaitre N, Sougakoff W, Truffot-Pernot C, Jarlier V. *Characterization of new mutations in pyrazinamide-resistant strains of Mycobacterium tuberculosis and identification of conserved regions important for the catalytic activity of the pyrazinamidase pncA. Antimicrob Agents Chemother 1999; 43: 1761-3.*
20. Marttila HJ, Marjamaki M, Vyshnevskaya E, Vyshnevskiy BI, Otten TF, Vasilyef AV, Viljanen MK. *pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis isolates from Northwestern Russia. Antimicrob Agents Chemother 1999; 43: 1764-6.*
21. Mestdagh M, Fonteyne PA, Realini L, Rossau R, Jannes G, Mijs W, De Smet KAL, Portaels F, van den Eeckhout E. *Relationship between pyrazinamide resistance, loss of pyrazinamidase activity, and mutations in the pncA locus in multidrug-resistant clinical isolates of Mycobacterium tuberculosis. Antimicrob Agents Chemother 1999; 43: 2317-9.*
22. Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, Zhang Y. *Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother 1997; 41: 540-3.*
23. Sreevatsan S, Pan X, Zhang Y, Kreiswirth BN, Musser JM. *Mutations associated with pyrazinamide resistance in pncA of Mycobacterium tuberculosis complex organisms. Antimicrob Agents Chemother 1997; 41: 636-40.*
24. Park YK, Bai GH, Kim SJ. *Restriction fragment length polymorphism analysis of Mycobacterium tuberculosis isolated from countries in the Western Pacific region. J Clin Microbiol 2000; 38: 191-7.*
25. Wayne LG. *Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am Rev Respir Dis 1974; 109: 147-51.*
26. Brown TJ, Tansel O, French GL. *Simultaneous identification and typing of multi-drug-resistant Mycobacterium tuberculosis isolates by analysis of pncA and rpoB. J Med Microbiol 2000; 49: 651-6.*
27. Butler WR, Kilburn JO. *Susceptibility of Mycobacterium tuberculosis to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob Agents Chemother 1983; 24: 600-1.*
28. Zhang Y, Scorpio A, Nikaido H, Sun Z. *Role of acid pH and deficient efflux of pyrazinoic acid in unique susceptibility of Mycobacterium tuberculosis to pyrazinamide. J Bateriol 1999; 181: 2044-9.*
29. Raynaud C, Laneelle MA, Senaratne RH, Draper P, Laneelle G, Daffe M. *Mechanisms of pyrazinamide resistance in mycobacteria: importance of lack of uptake in addition to lack of pyrazinamidase activity. Microbiol 1999; 145: 1359-67.*
30. Speirs RJ, Welch JT, Cynamon MH. *Activity of n-propyl pyrazinoate against pyrazinamide-resistant Mycobacterium tuberculosis: investigations into mechanism of action of and mechanism of resistance to pyrazinamide. Antimicrob Agents Chemother 1995; 39: 1269-71.*
31. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T. *Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis. Lancet 1993; 341: 647-50.*
32. Derst C, Henseling J, Rohm KH. *Engineering the substrate specificity of Escherichia coli asparaginase. II. Selective reduction of glutaminase activity by amino acid replacements at position 248. Protein Sci 2000; 9: 2009-17.*
33. Sideraki V, Huang W, Palzkill T, Gilbert HF. *A secondary drug resistance mutation of TEM-1 beta-lactamase that suppresses misfolding and aggregation. Proc Natl Acad Sci USA 2001; 98: 283-8.*
34. Creighton TE. *Proteins: Structure and molecular properties. Freeman and Co., New York, N. Y. 1984; 159-97.*
35. Espinosa de les Moteros LE, Galan JC, Gutierrez M, Samper S, Garcia Marin JF, Martin C, Dominguez L, Rafael L, Baquero F, Gomez-Mampaso E, Blazquez J. *Allele-specific PCR method based on pncA and oxyR sequences for distinguishing Mycobacterium bovis from Mycobacterium tuberculosis: intraspecific M. bovis pncA sequence polymorphism. J Clin Microbiol 1998; 36: 239-42.*
36. Scorpio A, Collins D, Whipple D, Cave D, Bates J, Zhang Y. *Rapid differentiation of bovine and human tubercle bacilli based on a characteristic mutation in the bovine pyrazinamidase gene. J Clin Microbiol 1997; 35: 106-10.*
37. Escalante P, Ramaswamy S, Sanabria H, Soini H, Pan X, Valiente-Castillo O, Musser JM. *Genotypic characterization of drug-resistant Mycobacterium tuberculosis isolates from Peru. Tuber Lung Dis 1998; 79: 111-8.*
38. Zhang Y, Heym B, Allen B, Young D, Cole S. *The catalase-peroxi-*

dase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature 1992; 358: 591-3.

39. Lewin B. *Genes VII. 2000. Oxford University Press Inc., New York, N. Y. 2000; 147-9.*
40. Bifani PJ, Plikaytis BB, Kapur V, Stockbauer K, Pan X, Lutfey ML, Moghazeh SL, Eisner W, Daniel TM, Kaplan MH, Crawford JT, Musser JM, Kreiswirth BN. *Origin and interstate spread of a New York city multidrug-resistant Mycobacterium tuberculosis clone family. JAMA 1996; 275: 452-7.*
41. Gutierrez MC, Galan JC, Blazquez J, Bouvet E, Vincent V. *Molecular markers demonstrate that the first described multidrug-resistant Mycobacterium bovis outbreak was due to Mycobacterium tuberculosis. J Clin Microbiol 1999; 37: 971-5.*