Characterization of \textit{pncA} Mutations of Pyrazinamide-Resistant \textit{Mycobacterium tuberculosis} in Korea

Pyrazinamide (PZA) is one of the most important drugs for the treatment of \textit{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 \textit{pncA} 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 \textit{pncA} 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 \textit{pncA} mutations observed in this study supports the usefulness of DNA-based detection of PZA-resistant \textit{M. tuberculosis}. Having verified the scattered and diverse mutational characteristics of the \textit{pncA} gene, automated DNA sequencing seems to be the best strategy for rapid detection of PZA-resistant \textit{M. tuberculosis}.

\textbf{Key Words}: Mycobacterium tuberculosis; Drug Resistance; Pyrazinamide, \textit{pncA}; Mutation
originated 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 stop 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 entire 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 MgCl₂, 0.01% (w/v) gelatin, 10 mM beta-mercaptoethanol, and 10% (v/v) DMSO. The reaction mixtures were subjected to 5 min at 96°C, followed by 35 cycles of 20 sec at 96°C, 30 sec at 55°C, 60 sec at 72°C and terminated by an additional 10 min at 72°C. Successful gene amplifications were confirmed by UV transillumination following electrophoresis on a 1% agarose gel stained with ethidium bromide in 1 x TAE buffer.

DNA sequencing analysis

PCR products from each M. tuberculosis strain were purified with the PCR purification kit (Qiagen Inc., Valencia, CA, U.S.A.), 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.
Mutations in pncA

Mutations identified in PZA-resistant M. tuberculosis isolates* in Korea

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

| Changes                        | Nucleotide substitution (position) | Amino acid substitution (residue) | No. of isolates |
|--------------------------------|-----------------------------------|-----------------------------------|-----------------|
| C→T (134)*                    | Gln→stop (10)                     |                                   | 1               |
| A→C (137)                     | Gln→Pro (10)                      |                                   | 3               |
| A→G (139)                     | Gln→Arg (10)                      |                                   | 1               |
| A→G (146)                     | Thr→Ala (12)                      |                                   | 1               |
| T→C (172)                     | Pro→Leu (58)                      |                                   | 1               |
| T→C (190)                     | Tyr→Asp (64)                      |                                   | 1               |
| T→C (199)                     | Ser→Pro (67)                      |                                   | 1               |
| T→G (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)                     | Gln→Asp (78)                      |                                   | 1               |
| T→G (254)                     | Leu→Arg (85)                      |                                   | 1               |
| T→G (254)                     | Leu→Pro (85)                      |                                   | 1               |
| A→C (286)                     | Lys→Gln (96)                      |                                   | 1               |
| A→G (286)                     | Lys→Glu (96)                      |                                   | 3               |

*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.
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 shifts, 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 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).

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REFERENCES

1. Pablos-Mendez A, Raviglione MC, Laszlo A, Binkin N, Rieder HL, Bustreo F, Cohn DL, Lambregts-van Wezenbeek 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, Fujiiwara PL, 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, Tomssett R. Activation of pyrazinamide and nicotinamide in acidic environment in vitro. Am Rev Respir Dis 1970; 142: 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
6 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. Tuberlcle 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. Tuberc Lung Dis 1997; 78: 117-22.

19. Lemaire N, Sougakoff W, Truffot-Pernot C, Jarlier V. Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. J Antimicrob Chemother 1997; 40: 119-27.

20. Marttila HJ, Marjamaki M, Viikinko-Aho M, Marjamaki M. Pyrazinamidase activity of Mycobacterium tuberculosis-resistant Mycobacterium tuberculosis complex organisms. Antimicrob Agents Chemother 1997; 41: 636-40.

21. Park YK, Bai GH, Kim SJ. Restriction fragment length polymorphism analysis of Mycobacterium tuberculosis isolates from countries in the Western Pacific region. J Clin Microbiol 2000; 38: 191-7.

22. Wayne LG. Simple pyrazinamidase and arese tests for routine identification of mycobacteria. Am Rev Respir Dis 1974; 109: 147-51.

23. 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.

24. Butler WR, Kilburn JO. Susceptibility of Mycobacterium tuberculosis to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob Agents Chemother 1983; 24: 600-1.

25. 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 Bacteriol 1999; 181: 2044-9.

26. Raynaud C, Laneelle MA, Sennarate 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.

27. Selenski R, 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.

28. Talenti 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.

29. 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.

30. 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.

31. Croom SP, Proctor ME, Dmytrenko VS, Pancholi KV, Walmsley SL, Horsley V, et al. The catalase-peroxidase gene cluster in Mycobacterium tuberculosis. FEMS Microbiol Lett 2001; 202: 267-73.

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 los Moteros LE, Galan JC, Gutierrez M, Samper S, Garcia Marin JF, Martin C, Domínguez 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 S, Soini H, Pan X, Valiente-Castillo O, Musser JM. Genotypic characterization of drug-resistant Mycobacterium tuberculosisisolates from Peru. Tuber Lung Dis 1998; 79: 111-8.

38. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxi-
pncA Mutations in *Mycobacterium tuberculosis*  

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, Luthey 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.