

Molecular characterization of *pncA* gene mutations in *Mycobacterium tuberculosis* clinical isolates from China

L. HOU^{1,2}, D. OSEI-HYIAMAN^{1,2*}, Z. ZHANG⁵, B. WANG⁶, A. YANG⁴
AND K. KANO³

¹Department of Medical Microbiology and Infectious Diseases, ²Graduate School of Medicine,

³Institute of Community Medicine, University of Tsukuba, Tsukuba City, Ibaraki-ken, Japan

⁴Shanxi Provincial Tuberculosis Hospital, Shanxi China

⁵Department of Epidemiology, Shanxi Medical University, Shanxi, China

⁶Medical Information Centre, Beijing Medical University, Beijing, China

(Accepted 20 October 1999)

SUMMARY

A sample of 35 pyrazinamide (PZA)-resistant and 30 PZA-susceptible clinical isolates recovered from Beijing and Taiyuan City, China were characterized by SSCP and sequence analysis for mutations in the *pncA* gene that encodes the *Mycobacterium tuberculosis* PZase. The purpose of this study was to understand the molecular basis and the characteristics of *pncA* gene mutations and its relation to PZA resistance in *M. tuberculosis* strains from China. Several mutations with base changes leading to amino acid substitutions were found in the PZA-resistant isolates. No mutations were seen in the 243 PZA-susceptible isolates. Among the 35 PZA-resistant isolates, 32 isolates (91·4%) had nucleotide substitutions, insertions and deletions that resulted in amino-acid substitution; or frameshifts in some strains. Other previously uncharacterized mutations were found as follows: Asn118→Thr, CG insertion at position 501; CC insertion at nucleotide position 403; a 8 base-pair deletion at start codon; Pro54→Thr; AG insertion at 368; Tyr41→His, Ser88→stop, and A insertion at nucleotide position 301. IS6110 subtyping revealed that each strain was unique; indicative of the epidemiologic independence of the isolates.

INTRODUCTION

The resurgence of tuberculosis coupled with the emergence of drug-resistant *Mycobacterium tuberculosis* strains seriously compromises our ability to control tuberculosis [1]. Multiple drug-resistant strains of *M. tuberculosis* defined as resistance to at least isoniazid and rifampin have stimulated a great deal of research aimed at understanding the molecular basis of drug resistance in *M. tuberculosis*. Such knowledge should eventually facilitate the rational design of new anti-tuberculosis drugs; and the

development of rapid tests for proper detection of drug resistance.

Pyrazinamide (PZA); a nicotinamide analogue, has been in use for almost 50 years as a first line drug to treat tuberculosis. PZA is bactericidal to semi-dormant mycobacteria and reduces the total tuberculosis treatment time when used in combination with isoniazid and rifampin [2–9]. This has made PZA the third important drug in the list of modern therapy for tuberculosis. Although the exact biochemical basis of PZA activity *in vivo* is not known, under acidic conditions it is thought that the bacterial enzyme pyrazinamidase (PZase) is required to convert PZA to pyrazinoic acid (POA) which is toxic to *M. tuberculosis* [1, 2, 10, 11]. The finding that PZA-resistant

* Author for correspondence: Graduate School of Medicine, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba city, Ibaraki-ken, Japan 305-8575.

strains lose PZase activity and the hypothesis that PZase is required to convert PZA to POA intracellularly led to the recent cloning and characterization of the *M. tuberculosis* gene *pncA* that encodes PZase [2, 12]. DNA sequencing of PZA-resistant clinical isolates indicated mutations in the *pncA* gene: These mutations are currently thought to confer resistance to PZA. However, the extent of geographical variation in *pncA* gene mutations deserves further studies.

The present study sought to understand the molecular basis and characteristics of PZA resistance and its relation to potential *pncA* gene mutations in *M. tuberculosis* clinical isolates from China. We analysed the DNA sequence of the *pncA* gene in samples of PZA-resistant and PZA-susceptible clinical isolates from Beijing and Taiyuan, China.

METHODS

Mycobacterium strains

Thirty-five PZA-resistant and 30 PZA-susceptible clinical isolates recovered from Beijing and Taiyuan City, China, over a 2-year period (1996–8) were used in this study. All patients were inhabitants of Beijing and Taiyuan City, and 33 of the patients had previously received anti-tuberculosis therapy. Treatment history was unknown for three patients. The initial anti-tuberculosis therapy included isoniazid, rifampin, and pyrazinamide. All isolates included in this study were obtained before this regimen was changed based on the respective drug susceptibility results. All strains were characterized for their IS6110 subtypes by an internationally standardized protocol [13]. All isolates were tested for PZA susceptibility with either the BACTEC radiorespiratory method at pH 6.0 [14, 15] or by the conventional proportion method with a PZA concentration of 25 µg/ml in 7H10 agar (pH 5.5) [16]. Susceptibility was defined as MIC of no more than 100 µg/ml of PZA. Most of the strains analysed in this study were highly resistant to PZA (MIC ≥ 500 µg/ml). Re-testing for PZA susceptibility of PZA-resistant strains without *pncA* mutations was performed by both the BACTEC method and the 7H10 agar medium method at pH (5.5). Genomic DNA was prepared according to a protocol generously provided by Dr J. D. A. Van Embden [17]. All genomic DNA extraction and drug susceptibility tests were carried out at the Shanxi Medical University, Taiyuan, China; and then

shipped to the University of Tsukuba, Ibaraki, Japan, for further molecular analyses. Mycobacterium culturing and DNA isolation were conducted in Biosafety level 3 laboratory.

PCR conditions

Three sets of primers were designed according to the *Mycobacterium tuberculosis pncA* sequence (558 bp) (GenBank accession number U59967) [12] (Table 1). PCR was performed as described by Dr J. D. A. Van Embden [17]. The PCR conditions were 96 °C for 3 min, followed by 30 cycles of 96 °C for 1 min, 63 °C for 1 min and 72 °C for 2 min, with a final extension of 72 °C for 6 min.

SSCP analysis

SSCP was carried out as below; Briefly, PCR products (0.5–1 µg of DNA) were mixed with 6 µl of denaturing reaction solution (Perkin–Elmer), the mixture was denatured by boiling for 10 min, and cooled on ice for 5 min. The single-strand PCR products were loaded onto a 10% polyacrylamide gel. Electrophoresis was performed at 10 V/cm for 5 h at 10 °C. Single-strand bands were visualized by the ethidium bromide staining method.

Automated DNA sequencing of the *pncA* gene

The *pncA* genes were amplified by PCR using the conditions and primers described above. PCR products from isolates showing variant banding patterns in SSCP were cut from the gel and purified with the GeneClean kit (United State Biochemicals). The purified PCR products were directly sequenced using Genetic Analyzer (Perkin–Elmer, ABI PRISM^{AM} 310). After termination dideoxy-cycle sequencing, the resulting data were assembled and edited using SeqEd v. 1.0.3 software (Applied Biosystems). The sequences were compared with published sequence of *pncA* from Genbank [12]. All mutations in the *pncA* structural gene were confirmed by re-sequencing.

RESULTS

All *Mycobacterium tuberculosis* complex clinical isolates studied had a C→T change at nucleotide 67 which has been identified as a sequence error by

Table 1. Oligonucleotide primers for amplification of the pncA gene (558 bp)

| Primer | Sequence | Position |
|-----------|-----------------------------|------------------------------------|
| Primer P1 | 5'-GTCGGTCATGTTTCGCGATCG-3' | From -105 bp upstream |
| Primer P2 | 5'-TCGGCCAGGTAGTCGCTGAT-3 | Nucleotide position 110-91 |
| Primer P3 | 5'-ATCAGCGACTACCTGGCCGA-3' | Nucleotide position 91-110 |
| Primer P4 | 5'-GATTGCCGACGTGTCCAGAC-3' | Nucleotide position 270-25 |
| Primer P5 | 5'-CCACCGATCATTGTGTGCGC-3' | 401-420 |
| Primer P6 | 5'-GCTTTGCGGCGAGCGCTCCA-3' | 60 bp downstream of the stop codon |

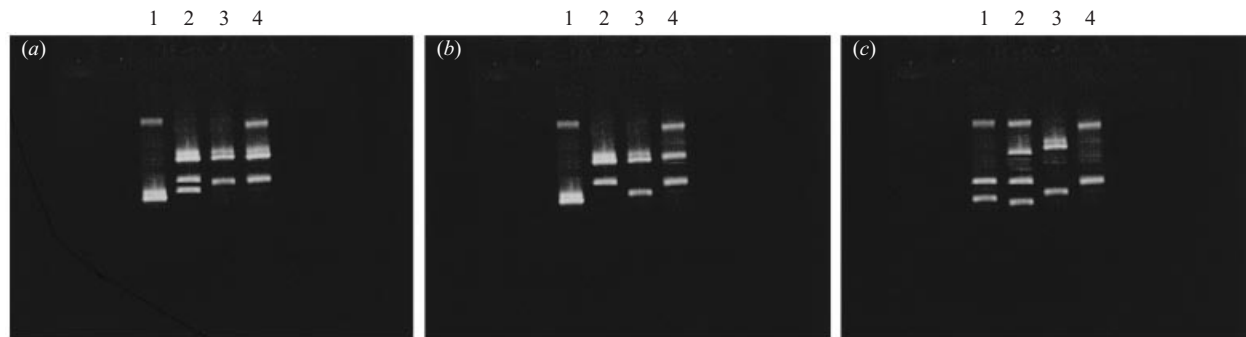


Fig. 1. PCR-SSCP analysis of *pncA* mutations in PZA-resistant *M. tuberculosis*. (A) SSCP with primer set 1. Lane 1, PZA-susceptible control strain CBS028. Lanes 2-4, PZA-resistant clinical isolates CBM04, CCT03, and CBC02, respectively. (B) SSCP analysis with primer set 2. Lane 1, PZA-susceptible control strain CBS028. Lanes 2-4, PZA-resistant clinical isolates CCT19, CBM06, and CCT01, respectively. (C) SSCP analysis with primer set 3. Lane 1, PZA-susceptible control strain CBS028. Lanes 2-4 PZA-resistant clinical isolates CBM01, CBM03, and CBM05, respectively.

Zhang and colleagues [9]: We will not consider them as mutations in this study. The results of IS6110 subtypes revealed that each was unique, indicative of the epidemiological independence of the isolates.

pncA gene in PZA-susceptible *M. tuberculosis* clinical isolates

PZA-susceptible isolates contained the wild-type *pncA* allele; no mutations were found in these isolates. These results indicate that mutations in the *pncA* gene are essential tools for the detection of PZA resistance.

PCR-SSCP analysis

PCR-SSCP analysis was performed for the rapid detection of point mutations in the *pncA* gene. Selected PZA-resistant clinical isolates were used for the PCR-SSCP analysis. This process was rapid and very convenient (Fig. 1).

pncA mutations in PZA-resistant *M. tuberculosis* clinical isolates

Results of the direct sequence analysis of *pncA* gene from different PZA-resistant isolates are shown in

Table 2. Among the 35 PZA-resistant clinical isolates, 32 isolates had mutations that included nucleotide substitutions (missense mutation), or insertions and deletions (nonsense mutation) leading to amino-acid substitutions in some cases; or frameshifts with corresponding nonsense polypeptides in some strains. The mutations found were diverse and dispersed along the entire *pncA* gene. Most of the mutations (68.8%, 22/32) are point mutations causing amino-acid substitutions (Table 2). For instance isolate CBC01 had a C to A change at nucleotide position 425, and a resulting Thr142→Lys substitution; CBC02 had A to C change at position 35, leading to an Asp12→Ala amino-acid substitution; isolate CBC03 had A to C change at nucleotide position 353 and caused a Asn118→Thr amino-acid substitution. Isolate CBC07 had a C deletion mutation at nucleotide position 28; CCT03 had C deletion at 59; CCT08 had G deletion at 71, and CCT16 had a C deletion at 28. All deletion mutations caused amino acid frameshifts. CBM01 had CG insertion mutation at position 501 and CBM02 had CC insertion mutation at 403, leading to amino-acid frameshifts. Two isolates had a mutation that created a stop codon; CBM06 had C→T change at nucleotide position 123, causing a change of Tyr41→stop. CCT19 had C to A change at

Table 2. *Epidemiological data and mutations observed among clinical isolates*

| Isolates | PZA MIC ($\mu\text{g/ml}$) | Base change | Amino-acid substitution | Patients' data | | | |
|----------|------------------------------|---------------------------------|-------------------------|----------------|--------|--------------------------|------------------------------------|
| | | | | Age (yr) | Gender | History of PZA treatment | Associated drug-resistant profile* |
| CBC01 | 300 | C→A at 425 | Thr142→Lys | 45 | Male | Yes | HRS |
| CBC02 | 1500 | A→C at 35 | Asp 12→Ala | 34 | Female | Yes | HRES |
| CBC03 | 900 | A→C at 353 | Asn118→Thr | 57 | Female | Yes | S |
| CBC04 | 1500 | T→C at 56 | Leu19→pro | 76 | Male | Unknown | HR |
| CBC06 | 1000 | T→C at 416 | Val139→Ala | 21 | Male | Yes | HRS |
| CBC07 | 400 | Nucleotide C deletion at 28 | | 19 | Male | Yes | HRES |
| CBC09 | >2000 | C→G at 123 | Tyr41→stop | 43 | Male | No | HRES |
| CBM01 | 900 | CG insertion at position 501 | | 38 | Female | Yes | SHE |
| CBM02 | 1000 | CC insertion at 403 | | 18 | Male | Yes | RE |
| CBM03 | 500 | C→A at 425 | Thr142→Lys | 23 | Male | Yes | RHE |
| CBM04 | 1200 | 11-bp deletion at start | | 35 | Female | Unknown | RHES |
| CBM05 | 600 | G→A at 413 | Cys138→Tyr | 62 | Female | Yes | RHES |
| CBM06 | 1500 | C→G at 123 | Tyr41→stop | 57 | Male | Yes | RSE |
| CCT01 | 900 | A→C at 139 | thr47→Pro | 19 | Female | Yes | SHE |
| CCT02 | >2000 | 8-bp deletion at start position | | 39 | Female | Yes | RHSE |
| CCT03 | 1800 | C deletion at position 59 | | 57 | Male | Yes | RSE |
| CCT04 | 900 | G→T at 288 | Lys96→Asn | 45 | Male | No | RSHE |
| CCT05 | 600 | C→A at 425 | Thr142→Lys | 35 | Female | Yes | HS |
| CCT06 | 800 | C→A at 160 | Pro54→Thr | 56 | Male | Yes | RHES |
| CCT07 | 1200 | T→G at 254 | Pro69→Arg | 34 | Male | Unknown | HRES |
| CCT08 | 600 | G deletion at 71 | | 56 | Male | Yes | RH |
| CCT09 | 900 | G→T at 288 | Lys96→Asn | 35 | Male | Yes | HRS |
| CCT11 | 1000 | T→C at 416 | Val139→Ala | 36 | Male | Yes | HS |
| CCT12 | 1000 | G→C at 362 | Arg121→Pro | 71 | Male | Yes | HRES |
| CCT13 | 1200 | AG insertion at 368 | | 68 | Female | Yes | RHES |
| CCT14 | 800 | T→C at 254 | Leu85→Pro | 34 | Female | Yes | RE |
| CCT15 | 900 | G→C at 415 | Val139→Leu | 45 | Male | Yes | RHES |
| CCT16 | 1500 | Nucleotide C deletion at 28 | | 58 | Female | Yes | HRS |
| CCT17 | 600 | T→C at 121 | Tyr41→His | 76 | Male | Yes | HRE |
| CCT18 | 1500 | T→C at 214 | Cys72→Arg | 69 | Female | Yes | HRES |
| CCT19 | >2000 | C→A at 263 | Ser88→Stop | 45 | Male | Yes | HRES |
| CCT20 | 1000 | A insertion at 301 | | 67 | Female | Yes | HRS |

* H, isonizaid; R, rifampin; S, streptomycin; E, ethambutol.

263, leading to a change of Ser88→stop. Two isolates had deletion mutations at start codon; CCT02 had 8-bp deletion and CBM04 had 11-bp deletion. These mutations may weaken the promoter activity and thus decrease the level of PZase expression in such mutants. Overall, 26 types of mutations were found. However,

a certain degree of conservation of *pncA* mutations were also observed at the following nucleotides: CCT09 isolates and CCT04 had the same mutation at codon Lys96→Asn; CBC06, CCT11 had the same mutation of Val139→Ala; and CBM03, CBC01 and CCT05 had the same mutation of Thr142→Lys.

Other previously uncharacterized mutations were also found, and includes the following changes: Asn118→Thr, CG insertion at position 501; CC insertion at 403; an 8 bp deletion at start codon; Pro54→Thr; AG insertion at 368; Tyr41→His, and Ser88→stop and A insertion at 301. Several other mutations previously identified by Hirano and colleagues were also found in our clinical isolates (Table 2) [18].

Three out of 35 PZA-resistant isolates did not contain any detectable *pncA* mutations (CBC05, CBC08 and CTT10). One of these isolates was actually susceptible to PZA; while two of them were resistant to PZA upon re-testing by both methods [14–16].

DISCUSSION

Recent studies have indicated that mutations in the *M. tuberculosis pncA* gene are associated with PZA resistance [12]. These studies have thus promoted a more thorough examination of sequence variations in this gene in order to identify genetically distinct susceptible and resistant organisms. In this study we found that 32 out of 35 PZA-resistant clinical *M. tuberculosis* isolates had mutations in the *pncA* gene. Unlike mutation in other *M. tuberculosis* genes such as *rpoB* and *KatG* genes, the spectrum of mutations in the *pncA* gene was very broad. A large number of distinct *pncA* gene mutations were associated with PZA resistance; and the changes were distributed virtually throughout the entire length of the gene. Most of the *pncA* gene mutations had nucleotide substitutions resulting in single amino-acid replacements. This discovery extends the common observation that has emerged from studies of antimicrobial resistance *M. tuberculosis* [1].

Our data also showed nucleotide deletion (18.8%; 6/32) and nucleotides insertion mutation (12.5%; 4/32). Changes resulting in frameshift mutations may be associated with PZA resistance. The results obtained in our study are supported by several published literatures [1, 12, 19]. Hirano and colleagues found a correlation of the development of PZA resistance with mutations in the *pncA* gene. The large number of distinct *pncA* gene mutations associated with PZA resistance supports the notion that enzymatic activity against PZA can be influenced by more than one molecular mechanism [1].

Further studies are needed to assess the role of the specific mechanisms of the respective *pncA* gene mutations on PZA resistance, and the level of PZase

activity alterable by the different mutations. The results of this study also indicate that regular survey of clinical isolates with a combination of PCR–SSCP analysis could help not only in epidemiological studies of *M. tuberculosis* outbreaks, but also in understanding the rate and trends in mutations; in relation to PZA resistance over time. In this study we observed a possible association between single nucleotide changes leading to frameshift mutations and PZA resistance. Similar changes were found by Srinand and colleagues [1]; the occurrence of these mutations could probably influence the survival of the organisms. For instance, in their study, Srinand and colleagues observed that deletions at nucleotide 70 conferred to the organism an ability to maintain viability in the total absence of PZase activity [1].

Obviously, additional mechanisms of PZA have been known to exist. However, such mechanisms have not been fully elucidated. The most up-to-date study on alternate mechanisms was by Raynaud and colleagues in 1999; they showed that the uptake of PZA is required for the activation of the pro-drug. Also, an ATP-dependent transport system is probably involved in the uptake of PZA. Diffusion experiments by Raynaud and colleagues also showed that PZA diffusion through membrane bilayers was faster than glycerol in liposomes; whereas the presence of ompATb, a porin-like protein of *Mycobacterium tuberculosis* in proteoliposomes slightly increased the diffusion of PZA. Their study also indicated that only naturally PZA-susceptible species exhibited both PZase activity and PZA uptake; whilst no such correlation was observed in naturally resistant species [11]. Thus, a functional PZase and a PZA transport system are necessary for the susceptibility of *Mycobacterium tuberculosis*. Additionally, the lack of *pncA* gene mutations among the PZA-susceptible isolates is a clear indication of the utility of the methods applied in this study and elsewhere for differentiating susceptible and resistant clinical isolates [1, 12, 19].

It remains to be seen however, how the PCR–SSCP method can be accessible to clinical laboratories for rapid PZA susceptibility analysis, considering the economic costs and its combined portability.

ACKNOWLEDGEMENTS

H. L. and D. O. H. are supported by the Asia International Scholarship Foundation and by the Honjo International Scholarship Foundation, respectively. We gratefully acknowledge the assistance and

cooperation of the faculty and staff of the Shanxi Medical University, Taiyuan Public Health Bureau, Beijing Chest Hospital, 309 Chest Hospital, Beijing; and the Shanxi Provincial Tuberculosis Hospital, Taiyuan City, China. We thank Dr Harada Shoji and Mrs Nakamura of the Institute of Community Medicine, University of Tsukuba, Japan for their generous technical assistance. Our sincere gratitude to Dr Van Embden for providing us with the complete protocol for *Mycobacterium tuberculosis*.

REFERENCES

1. Srinand S, Pan X, Zhang Y, Kreiswirth BN, et al. Mutation associated with pyrazinamide resistance in *pncA* of *Mycobacterium tuberculosis* complex organisms. *Antimicrob Chemother* 1997; **636**–40.
2. Konno K, Feldmann FM, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. *Am Rev Respir Dis* 1967; **95**: 461–9.
3. Yeage RL, Munroe WGC, Dessau FI. Pyrazinamide (Aldinamide) in the treatment of pulmonary tuberculosis. *Am Rev Tuberc* 1952; **65**: 523–34.
4. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity in vitro against semidormant *Mycobacterium tuberculosis* bacterial population. *Am Rev Respir Dis* 1992; **145**: 1223–5.
5. Dickinson JM, Mitchison DA. Observations in vitro on the suitability of pyrazinamide for intermittent chemotherapy of tuberculosis. *Tubercle* 1970; **51**: 389–96.
6. Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. *Tubercle* 1985; **66**: 219–25.
7. Singapore Tuberculosis Service/British Medical Research Council. Clinical trial of six-month and four-month regimens of chemotherapy in the treatment of pulmonary tuberculosis: the results up to 30 months. *Tubercle* 1981; **62**: 95–102.
8. Snider DE, Rogowski J, Zierski M, Bek E, Long MW. Successful intermittent treatment of smear-positive pulmonary tuberculosis in six months. *Am Rev Respir Dis* 1982; **125**: 265–7.
9. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* 1992; **358**: 591–3.
10. Kapur V, Li LL, Hamrick MR, Plikaytis BB, et al. Rapid *Mycobacterium* species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing. *Arch Pathol Lab Med* 1995; **119**: 131–8.
11. Raynaud C, Laneelle MA, Senaratne RH, Draper P, Laneelle G, Mamadou D. Mechanisms of pyrazinamide resistance in *Mycobacterium*: importance of lack of uptake in addition to lack of pyrazinamide activity. *Microbiology* 1999; **145**: 1359–67.
12. 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.
13. Van Embden JDA, Cave JT, Crawford JW, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 1993; **31**: 406–9.
14. Heifets LB, Good RC. Current laboratory methods for the diagnosis of tuberculosis. In: Bloom BR, ed. *Tuberculosis: pathogenesis, protection, and control*. Washington, D.C.: American Society for Microbiology 1994: 85–110.
15. Salfinger M, Heifets LB. Determination of pyrazinamide MICs for *Mycobacterium tuberculosis* at different pHs by the radiometric method. *Antimicrob Ag Chemother* 1988; **32**: 1002–4.
16. Stottmeier S, Beam RE, Kubica GP. Determination of drug susceptibility to pyrazinamide in 7H10 agar. *Am Rev Respir Dis* 1967; **96**: 1072–5.
17. Butler WR, Kilburn JO. Improved method for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *J Clin Microbiol* 1982; **16**: 1106–9.
18. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutations in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. *Tubercle Lung Dis* 1998; **78**: 117–22.
19. Scorpio A, Lingholm-Levy P, Heifets L, et al. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. *Antimicrob Ag Chemother* 1997: 540–3.