Evaluation of a line probe assay for the rapid detection of *gyrA* mutations associated with fluoroquinolone resistance in multidrug-resistant *Mycobacterium tuberculosis*

Hiroki Ando,¹ Satoshi Mitarai,² Yuji Kondo,³ Toshinori Suetake,³ Seiya Kato,² Toru Mori² and Teruo Kirikae¹

Correspondence

Teruo Kirikae

tkirikae@ri.ncgm.go.jp

¹Department of Infectious Diseases, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan

²Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, 3-1-24 Matsuyama, Kiyose, Tokyo 204-8533, Japan

³Third Department, Research and Development Laboratory, Nipro Corporation, 3023 Noji, Kusatsu, Shiga 525-0055, Japan

The aim of this study was to establish the importance of detecting fluoroquinolone (FQ) resistance in multidrug resistant (MDR) *Mycobacterium tuberculosis*, and to show the usefulness of a hybridization-based line probe assay (LiPA) for detecting *gyrA* mutations. Thirty-three MDR *M. tuberculosis* isolates were collected from a total of sixty MDR isolates identified in Japan over 6 months during a national surveillance study in 2002. Seventeen MDR isolates were collected by the National Center for Global Health and Medicine in Japan over 6 years from 2003 to 2008. These 50 isolates were examined for FQ susceptibility, and analysed by LiPA and *gyrA* sequencing. Among them, 22 (44 %) showed FQ resistance. All FQ-resistant isolates had at least one mutation in *gyrA*. The results of the LiPA were fully consistent with the DNA sequencing results. Given that on the basis of our results almost half of the MDR *M. tuberculosis* isolates in Japan might have resistance to FQ, it is important to monitor FQ resistance in patients with MDR tuberculosis (TB), as well as with drug-susceptible TB, prior to commencing treatment. For the detection of FQ resistance, LiPA is useful and can rapidly and efficiently assess FQ resistance.

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INTRODUCTION

The emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis* are a serious threat to the control of tuberculosis (TB), and constitute an increasing public-health problem (Caminero, 2010; Johnston *et al.*, 2009; Sotgiu *et al.*, 2009; Van Deun *et al.*, 2010a). Multidrug resistant (MDR) strains are defined as strains resistant to both rifampicin and isoniazid, and extensively drug-resistant strains are defined as MDR plus resistance to any fluoroquinolone (FQ) and to at least one of the second-line injectable drugs. Patients infected with these strains are difficult to cure and are more likely to remain infected for a longer period of time than patients with drug-susceptible strains (Caminero, 2010; Johnston *et al.*, 2009; Sotgiu *et al.*,

2009). With the emergence of MDR and extensively drug-resistant TB, the need for rapid drug susceptibility testing is now apparent (Van Deun *et al.*, 2010a).

FQ is a class of antimicrobial drug with strong activity against *M. tuberculosis* and is one of the most important second-line antituberculosis drugs used in the treatment of MDR TB (Yew *et al.*, 2010). Newer FQs, including gatifloxacin (GFLX) and moxifloxacin (MFLX), have been developed. A prospective study (Van Deun *et al.*, 2010b) indicated that a treatment regimen including GFLX was highly effective for MDR TB. A phase II trial study (Conde *et al.*, 2009) showed that a regime including MFLX improved culture conversion in the initial treatment of TB.

The main target of FQ in *M. tuberculosis* is the DNA gyrase encoded by *gyrA* and *gyrB*, which is essential for DNA supercoiling (Guillemin *et al.*, 1998). The genetic mechanism of FQ resistance is mainly due to alterations in the DNA gyrase, especially mutations in a short sequence called the quinolone-resistance-determining region (QRDR) of the *gyrA* (Guillemin *et al.*, 1998).

Abbreviations: CPFX, ciprofloxacin; FQ, fluoroquinolone; GFLX, gatifloxacin; JATA, Japan Anti-Tuberculosis Association; LiPA, line probe assay; LVFX, levofloxacin; MDR, multidrug resistant; MFLX, moxifloxacin; NCGM, National Center for Global Health and Medicine; QRDR, quinolone-resistance-determining region; SPFX, sparfloxacin; TB, tuberculosis; TRC, Tuberculosis Research Committee.

In this study, 50 MDR *M. tuberculosis* isolates collected in Japan were examined for FQ susceptibility and analysed by *gyrA* sequencing. Furthermore, we developed and evaluated a hybridization-based line probe assay (LiPA) for the rapid detection of *gyrA* mutations, which can easily be used in clinical applications. The whole procedure takes only 9 h, and the estimated cost per sample is £22 (US \$35).

We found that nearly half of our Japanese MDR *M. tuberculosis* isolates had resistance to FQ. Therefore, it is important to detect FQ resistance in patients with MDR TB, as well as with drug-susceptible TB, prior to commencing treatment.

METHODS

Bacterial strains. Among the 50 MDR *M. tuberculosis* isolates, 33 were obtained from patients with TB in 9 hospitals in Japan located in various regions – 1 in Hokkaido, 1 in Tohoku, 4 in Kanto, 2 in Kinki and 1 in Kyushu – during a national surveillance study [conducted from June to November 2002 by the Tuberculosis Research Committee (Ryoken), Japan] (TRC, 2007). The remaining 17 were obtained from patients diagnosed as MDR TB in the National Center for Global Health and Medicine (NCGM) from 2003 to 2008. During the national surveillance study, 60 MDR *M. tuberculosis* isolates were obtained (TRC, 2007). Of them, 33 were recovered from bacterial stocks and used in this study. *M. tuberculosis* strain H37Rv (ATCC 27294) and a *Mycobacterium bovis* strain (BCG Japanese strain 172), which are susceptible to FQ, were used in this study. To determine the species specificity of the LiPA, the following 18 bacterial strains were also used: *Mycobacterium avium* (ATCC 25291), *Mycobacterium chelonae* (ATCC 19237), *Mycobacterium fortuitum* (RIMD 1317004, ATCC 15754), *Mycobacterium intracellulare* (JCM 6384, ATCC 13950), *Mycobacterium kansasii* (JCM 6379, ATCC 124878), *Mycobacterium nonchromogenicum* (JCM 6364, ATCC 124878), *Mycobacterium scrofulaceum* (JCM 6381, ATCC 19981), *Mycobacterium smegmatis* (ATCC 19420), *Mycobacterium terrae* (GTC 623, ATCC 15755), *Escherichia coli* (ATCC 8739), *Haemophilus influenzae* (IID 984, ATCC 9334), *Klebsiella pneumoniae* (IID 5209, ATCC 15755), *Legionella pneumophila* (GTC 745), *Mycoplasma pneumoniae* (IID 817), *Pseudomonas aeruginosa* (ATCC 27853), *Rhodococcus equi* (ATCC 33710), *Staphylococcus aureus* strain N315 and *Streptococcus pneumoniae* (GTC 261).

FQ susceptibility testing. Drug susceptibility testing was performed at two institutions [33 isolates at the Japan Anti-Tuberculosis Association (JATA) and 17 at the NCGM]. Susceptibility to levofloxacin (LVFX), sparfloxacin (SPFX) and ciprofloxacin (CPFX) was determined at JATA, and susceptibility to LVFX was determined at the NCGM. A broth dilution method (BrothMIC MTB-I; Kyokuto) was used at JATA, and an agar proportion method with egg-based Ogawa medium (Vit Spectrum-SR; Kyokuto) was used at the NCGM. The proportion method is based on a slight modification of the World Health Organization protocol (Aziz *et al.*, 2003) and is recommended by the Japanese Society for Tuberculosis (Aziz *et al.*, 2003; Fujiki, 2001). The results are shown in Table 1.

Isolation of genomic DNA. Genomic DNA was extracted from bacteria as described previously (Sekiguchi *et al.*, 2007b).

PCR and DNA sequencing. The QRDR of *gyrA* was amplified and sequenced by a previously described method (Sekiguchi *et al.*, 2007a). DNA sequences were compared with H37Rv using GENETYX-MAC, version 14.0.2 (Genetix).

LiPA. The LiPA was performed as described previously (Ando *et al.*, 2010; Sekiguchi *et al.*, 2007b). Seven oligonucleotide probes were designed for the LiPA to detect *gyrA* mutations (Table 2). Two oligonucleotide probes, S1 and S2, were designed to cover the entire QRDR of *gyrA* of H37Rv. S2 also contained another probe to compensate for a neutral mutation of G to C at nucleotide position 284 (S95T) (Zhang & Telenti, 2000). The remaining five oligonucleotide probes, R1–R5, were designed to detect five mutations that were reported previously in FQ-resistant isolates obtained in Japan (Sekiguchi *et al.*, 2007a). These probes were immobilized on strips of nitrocellulose membrane. The QRDR of *gyrA* was amplified by nested PCR. Immobilized probes were hybridized with the biotinylated PCR products, and then incubated with streptavidin labelled with alkaline phosphatase. The results were visualized using colour development after incubation with 5-bromo-4-chloro-3'-indolyl-phosphate *p*-toluidine and nitro blue tetrazolium. The presence or absence of bands on all strips was determined visually. Among the 20 bacterial strains described above, 2 strains of *M. tuberculosis* and *M. bovis* were positive for PCR, but the other 18 strains were negative. The PCR products from the two strains hybridized with the S probes, but not the R probes (data not shown). These data indicated that the LiPA is specific for *M. tuberculosis* and *M. bovis*. The sensitivity of the LiPA was 24.2 fg *M. tuberculosis* DNA, equivalent to five copies of *gyrA* (data not shown).

RESULTS AND DISCUSSION

Fifty MDR *M. tuberculosis* isolates were collected. Of them, 33 were collected from a total of 60 MDR isolates identified in Japan over 6 months during the national surveillance study in 2002, and 17 were collected in the NCGM in Japan over 6 years from 2003 to 2008. These isolates and H37Rv were examined for FQ susceptibility, and 22 (44 %) of the isolates were resistant to FQ (Table 1). Among the 33 MDR isolates analysed at JATA, 14 (42 %) were resistant to all three FQs used, i.e. LVFX, SPFX and CPFX. None of the isolates were resistant to only one or two of these FQs (Table 1). The MICs of the three FQs for FQ-susceptible isolates ranged from 0.06 to 0.5 mg l⁻¹ (median 0.125 mg l⁻¹) for LVFX, from 0.03 to 0.25 mg l⁻¹ (median 0.125 mg l⁻¹) for SPFX, and from 0.06 to 0.5 mg l⁻¹ (median 0.25 mg l⁻¹) for CPFX. The MICs for FQ-resistant isolates ranged from 2 to 16 mg l⁻¹ (median 4 mg l⁻¹) for LVFX, from 1 to 8 mg l⁻¹ (median 4 mg l⁻¹) for SPFX, and from 2 to 16 mg l⁻¹ (median 8 mg l⁻¹) for CPFX. Among the 17 MDR isolates analysed at the NCGM, 8 (47 %) were resistant to LVFX (Table 1). There were no differences in rates of FQ resistance in MDR isolates between the two institutions (*P*=0.4).

We examined whether the MDR isolates had mutations in the QRDR of *gyrA* by LiPA and DNA sequencing analysis. Hybridization signals visualized as violet bands on the strips were strong and readily discernible with low background (data not shown). As shown in Table 1, regarding the FQ-resistance profile, the LiPA yielded results that were 100 % in agreement with those obtained by culture-based susceptibility testing. Of the 50 isolates, 28 were positive for both S1 and S2, and the remaining 22 were negative for one of the S probes (Δ S1 or Δ S2 in Table 1). Of the 22, 4 were positive for R1 indicating that isolates

Table 1. FQ susceptibility, LiPA profiles and *gyrA* mutations among 50 MDR *M. tuberculosis* isolates

Source of isolate	Strain	FQ MIC (mg l ⁻¹)			LiPA profile*	Mutation	
		LVFX†	SPFX	CPFX		Nucleotide change	Amino acid change
Control strain	H37Rv	0.25	0.125	0.25	WT (S1, S2)	—	—
JATA	2A-3-14	0.25	0.125	0.25	WT (S1, S2)	—	—
	2A-3-47	0.25	0.125	0.25	WT (S1, S2)	—	—
	2A-3-84	0.25	0.125	0.25	WT (S1, S2)	—	—
	2A-3-142	0.25	0.125	0.25	WT (S1, S2)	—	—
	2A-4-30	0.06	0.03	0.06	WT (S1, S2)	—	—
	2A-4-138	0.25	0.125	0.25	WT (S1, S2)	—	—
	2B-7-38	0.5	0.25	0.5	WT (S1, S2)	—	—
	2C-1-3	0.25	0.125	0.25	WT (S1, S2)	—	—
	2E-1-3	0.25	0.125	0.25	WT (S1, S2)	—	—
	2E-1-93	0.25	0.125	0.25	WT (S1, S2)	—	—
	2G-2-5	0.25	0.125	0.25	WT (S1, S2)	—	—
	2G-3-24	0.125	0.06	0.125	WT (S1, S2)	—	—
	2I-11-4	0.25	0.125	0.25	WT (S1, S2)	—	—
	2O-2-16	0.125	0.06	0.125	WT (S1, S2)	—	—
	2P-1-120	0.25	0.125	0.25	WT (S1, S2)	—	—
	2P-5-113	0.25	0.25	0.25	WT (S1, S2)	—	—
	2P-5-253	0.5	0.25	0.5	WT (S1, S2)	—	—
	2R-1-48	0.25	0.125	0.25	WT (S1, S2)	—	—
	2V-5-5	0.125	0.06	0.25	WT (S1, S2)	—	—
	2A-3-11	4	2	4	ΔS1, R1(A90V)	C269T	A90V
	2B-7-33	8	4	8	ΔS2, R4(D94G)	A281G	D94G
	2O-4-41	4	4	8	ΔS2, R5(D94A)	A281C	D94A
	2O-5-21	2	1	4	ΔS2, R5(D94A)	A281C	D94A
	2P-1-57	2	1	2	ΔS2, R5(D94A)	A281C	D94A
	2P-1-114	4	2	8	ΔS2, R4(D94G)	A281G	D94G
	2P-1-118	2	2	4	ΔS2, R5(D94A)	A281C	D94A
	2P-5-58	8	4	8	ΔS1, R1(A90V)	C269T	A90V
	2P-5-65	4	4	8	ΔS2, R4(D94G)	A281G	D94G
	2P-5-108	4	4	8	ΔS2, R4(D94G)	A281G	D94G
	2P-5-167	2	1	4	ΔS2, R5(D94A)	A281C	D94A
	2P-5-230	4	4	8	ΔS2, R4(D94G)	A281G	D94G
	2P-5-233	4	4	8	ΔS2, R5(D94A)	A281C	D94A
	2P-5-254	2	1	4	ΔS2, R5(D94A)	A281C	D94A
NCGM	NCGM2819	S	ND	ND	WT (S1, S2)	—	—
	NCGM2825	S	ND	ND	WT (S1, S2)	—	—
	NCGM2847	S	ND	ND	WT (S1, S2)	—	—
	NCGM2861	S	ND	ND	WT (S1, S2)	—	—
	NCGM2862	S	ND	ND	WT (S1, S2)	—	—
	NCGM2864	S	ND	ND	WT (S1, S2)	—	—
	NCGM2929	S	ND	ND	WT (S1, S2)	—	—
	NCGM2931	S	ND	ND	WT (S1, S2)	—	—
	NCGM2933	S	ND	ND	WT (S1, S2)	—	—
	NCGM2803	R	ND	ND	ΔS2	G280T	D94Y
	NCGM2822	R	ND	ND	ΔS2, R5(D94A)	A281C	D94A
	NCGM2834	R	ND	ND	ΔS1, R1(A90V)	C269T	A90V
	NCGM2835	R	ND	ND	ΔS2, R5(D94A)	A281C	D94A
	NCGM2863	R	ND	ND	ΔS2, R4(D94G)	A281G	D94G
	NCGM2888	R	ND	ND	ΔS2, R4(D94G)	A281G	D94G
	NCGM2930	R	ND	ND	ΔS2, R4(D94G)	A281G	D94G
	NCGM2932	R	ND	ND	ΔS1, R1(A90V)	C269T	A90V

ND, Not determined; R, resistant to LVFX; S, sensitive to LVFX; WT, wild-type.

*Δ indicates a negative signal for the probe.

†S and R results were determined by the agar proportion method.

Table 2. Locations of oligonucleotide probes designed to cover *M. tuberculosis gyrA*

Probe	Amino acid region detected by each probe	Nucleotide sequence*
S1	88–92	GATCGACGCGTCGCC
S2	92–97	CACCAGGSTGTCGTAGAT
R1	A90V mutation	GATCGAC A CGTCGCC
R2	D94G mutation	CACCAGGCTG C CGTAGAT
R3	D94A mutation	CACCAGGCTG G CGTAGAT
R4	D94G-S95T mutations	CACCAGG G TG C CGTAGAT
R5	D94A-S95T mutations	CACCAGG G TG G CGTAGAT

*S represents C or G; bold letters indicate mutations.

had an A90V mutation, 8 were positive for R4 (D94G mutation) and 9 were positive for R5 (D94A mutation). However, one isolate, NCGM2803, with Δ S2 was negative for R1–R5 indicating that the LiPA could not identify a mutation associated with FQ resistance. As shown in Table 1, the DNA sequencing data were fully consistent with results obtained by culture-based susceptibility testing. The FQ-resistant NCGM2803 isolate had a mutation of g280t (D94Y). In addition, it was reported that FQ-resistant isolates had mutations in *gyrB* but not in *gyrA* (Aubry *et al.*, 2006). Therefore, the present LiPA will be improved to detect the mutations.

The number of the MDR isolates used in this study is enough to allow estimation of the rate of FQ resistance in Japan. We tested 33 of 60 MDR isolates collected in the national surveillance study. JATA reported that there were 11 933 cases positive for sputum smear in Japan in 2002 (www.jata-org.jp). Whereas, 3122 isolates were collected for 6 months in 2002 during the national surveillance study (TRC, 2007). It is estimated that the number of isolates collected in the study covered more than 50 % of cases positive for sputum smear. Of the 3122 isolates, 60 isolates were reported to be MDR (TRC, 2007).

Occasional monitoring of the drug susceptibility of patients with MDR TB before and during chemotherapy is essential as MDR isolates can easily acquire resistance to additional antituberculosis drugs. We reported previously that more than 50 % of MDR isolates have already acquired PZA resistance (Ando *et al.*, 2010). In the present study, we found that nearly half of our Japanese MDR *M. tuberculosis* isolates were resistant to FQ. However, little information is available regarding resistance to other second-line drugs, and it will be necessary in future to monitor susceptibility to these drugs.

We strongly suggest that FQ susceptibility needs to be monitored as soon as patients are diagnosed with TB, because FQs are widely used for the treatment of bacterial infections. A population of patients with TB who received FQ treatment have been reported by Wang *et al.* (2006). Some other TB patients were initially treated as having community-acquired pneumonia and were administered FQs (Yoon *et al.*, 2005). We could not obtain information

about the TB patients whose isolates were tested in the present study, especially about their previous treatments with antituberculosis drugs, including FQs. Nevertheless, it is very likely that these patients received FQs. In Japan, FQs were used for patients with TB, especially MDR TB, before 2008 but LVFX, MFLX, GFLX, SPFX and CPFX were not officially recommended as antituberculosis drugs by the Japanese Society for Tuberculosis until 2008.

It is also necessary to develop a rapid and inexpensive diagnostic method to determine the drug susceptibility of *M. tuberculosis*. The whole procedure of the LiPA described here takes only 9 h, and the estimated cost per sample is £22 (US \$35). A DNA sequencing-based method (Sekiguchi *et al.*, 2007a) is also rapid, but is more expensive than the LiPA. Therefore, the LiPA is suitable for this purpose. Clinical trials for *in vitro* diagnosis including non-MDR TB are in progress in Japan.

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REFERENCES

- Ando, H., Mitarai, S., Kondo, Y., Suetake, T., Sekiguchi, J. I., Kato, S., Mori, T. & Kirikae, T. (2010). Pyrazinamide resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates in Japan. *Clin Microbiol Infect* **16**, 1164–1168.
- Aubry, A., Veziris, N., Cambau, E., Truffot-Pernot, C., Jarlier, V. & Fisher, L. M. (2006). Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: functional analysis of mutant enzymes. *Antimicrob Agents Chemother* **50**, 104–112.
- Aziz, M., Laszlo, A., Raviglione, M. C., Rieder, H. L., Espinal, M. A. & Wright, A. (2003). *Guidelines for Surveillance of Drug Resistance in Tuberculosis*. Geneva: World Health Organization.

- Caminero, J. A. (2010).** Multidrug-resistant tuberculosis: epidemiology, risk factors and case finding. *Int J Tuberc Lung Dis* **14**, 382–390.
- Conde, M. B., Efron, A., Lored, C., De Souza, G. R., Graca, N. P., Cezar, M. C., Ram, M., Chaudhary, M. A., Bishai, W. R. & other authors (2009).** Moxifloxacin versus ethambutol in the initial treatment of tuberculosis: a double-blind, randomised, controlled phase II trial. *Lancet* **373**, 1183–1189.
- Fujiki, A. (2001).** *TB Bacteriology Examination to Stop TB*. Tokyo: The Research Institute of Tuberculosis.
- Guillemin, I., Jarlier, V. & Cambau, E. (1998).** Correlation between quinolone susceptibility patterns and sequences in the A and B subunits of DNA gyrase in mycobacteria. *Antimicrob Agents Chemother* **42**, 2084–2088.
- Johnston, J. C., Shahidi, N. C., Sadatsafavi, M. & Fitzgerald, J. M. (2009).** Treatment outcomes of multidrug-resistant tuberculosis: a systematic review and meta-analysis. *PLoS ONE* **4**, e6914.
- Sekiguchi, J., Miyoshi-Akiyama, T., Augustynowicz-Kopec, E., Zwolska, Z., Kirikae, F., Toyota, E., Kobayashi, I., Morita, K., Kudo, K. & other authors (2007a).** Detection of multidrug resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* **45**, 179–192.
- Sekiguchi, J., Nakamura, T., Miyoshi-Akiyama, T., Kirikae, F., Kobayashi, I., Augustynowicz-Kopec, E., Zwolska, Z., Morita, K., Suetake, T. & other authors (2007b).** Development and evaluation of a line probe assay for rapid identification of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* strains. *J Clin Microbiol* **45**, 2802–2807.
- Sotgiu, G., Ferrara, G., Matteelli, A., Richardson, M. D., Centis, R., Ruesch-Gerdes, S., Tounoussova, O., Zellweger, J. P., Spanevello, A. & other authors (2009).** Epidemiology and clinical management of XDR-TB: a systematic review by TBNET. *Eur Respir J* **33**, 871–881.
- TRC (2007).** Drug-resistant *Mycobacterium tuberculosis* in Japan: a nationwide survey, 2002. *Int J Tuberc Lung Dis* **11**, 1129–1135.
- Van Deun, A., Martin, A. & Palomino, J. C. (2010a).** Diagnosis of drug-resistant tuberculosis: reliability and rapidity of detection. *Int J Tuberc Lung Dis* **14**, 131–140.
- Van Deun, A., Maug, A. K., Salim, M. A., Das, P. K., Sarker, M. R., Daru, P. & Rieder, H. L. (2010b).** Short, highly effective, and inexpensive standardized treatment of multidrug-resistant tuberculosis. *Am J Respir Crit Care Med* **182**, 684–692.
- Wang, J. Y., Hsueh, P. R., Jan, I. S., Lee, L. N., Liaw, Y. S., Yang, P. C. & Luh, K. T. (2006).** Empirical treatment with a fluoroquinolone delays the treatment for tuberculosis and is associated with a poor prognosis in endemic areas. *Thorax* **61**, 903–908.
- Yew, W. W., Lange, C. & Leung, C. C. (2010).** Treatment of tuberculosis update 2010. *Eur Respir J* (in press). doi: 10.1183/09031936.00033010
- Yoon, Y. S., Lee, H. J., Yoon, H. I., Yoo, C. G., Kim, Y. W., Han, S. K., Shim, Y. S. & Yim, J. J. (2005).** Impact of fluoroquinolones on the diagnosis of pulmonary tuberculosis initially treated as bacterial pneumonia. *Int J Tuberc Lung Dis* **9**, 1215–1219.
- Zhang, Y. & Telenti, A. (2000).** *Genetics of Drug Resistance in Mycobacterium tuberculosis*. Washington, DC: American Society for Microbiology.