Journal of Microbiology, Immunology and Infection (2016) 49, 439–444

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

BRIEF COMMUNICATION

Second-line anti-tuberculosis drug resistance and its genetic determinants in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates

Zofia Bakuła [a], Agnieszka Napiórkowska [b], Michał Kamiński [a], Ewa Augustynowicz-Kopeć [b], Zofia Zwolska [b], Jacek Bielecki [a], Tomasz Jagielski [a,*]

[a] *Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, 02-096 Warsaw, Poland*
[b] *Department of Microbiology, National Tuberculosis and Lung Diseases Research Institute, 01-138 Warsaw, Poland*

Received 3 October 2014; received in revised form 17 March 2015; accepted 18 April 2015
Available online 15 May 2015

KEYWORDS
MDR-TB;
second-line drugs;
sequence analysis;
tuberculosis (TB);
XDR-TB

Abstract *Background:* Mutations in several genetic loci have been implicated in the development of resistance to second-line anti-tuberculosis (TB) drugs (SLDs). The purpose of this study was to investigate the prevalence of resistance to SLDs and its association with specific mutations in multidrug-resistant (MDR) *Mycobacterium tuberculosis* clinical isolates.
Materials and methods: The study included 46 MDR-TB isolates. Mutation profiling was performed by amplifying and sequencing the following six genes: *gyrA*/*gyrB*, *rrs*, *tlyA*, and *ethA*/*ethR*, in which mutations are implicated in resistance of tubercle bacilli to ofloxacin (OFX), amikacin (AMK), capreomycin, and ethionamide (ETH), respectively.
Results: Of the strains analyzed, 14 (30.4%) showed resistance to at least one of the four SLDs tested. Mutations in the *gyrA* gene occurred in 34 (73.9%) strains, with the most common amino acid change being Ser95Thr. The Asp94Asn and Ala90Val substitutions in the *gyrA* were present exclusively in OFX-resistant strains, yet represented only 40% of all OFX-resistant strains. The only mutation in the *gyrB* gene was substitution Ser447Phe, detected in one OFX-resistant isolate. None of the AMK-resistant strains carried a mutation in the *rrs* gene. Mutations in the *ethA*/*ethR* loci were found in one ETH-resistant and 11 ETH-susceptible strains.

* Corresponding author. Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, I. Miecznikowa 1, 02-096 Warsaw, Poland.
E-mail address: t.jagielski@biol.uw.edu.pl (T. Jagielski).

http://dx.doi.org/10.1016/j.jmii.2015.04.003

Conclusions: The results of this study challenge the usefulness of sequence analyses of tested genes (except *gyrA*) for the prediction of SLD resistance patterns and highlight the need for searching other genetic loci for detection of mutations conferring resistance to SLDs in *M. tuberculosis*.

Introduction

The two most detrimental forms of drug-resistant tuberculosis (TB) are multidrug-resistant (MDR)-TB, defined as resistance to at least isoniazid (INH) and rifampicin (RMP), and extensively drug-resistant (XDR)-TB, defined as MDR-TB with additional resistance to second-line anti-TB drugs (SLDs), that is any fluoroquinolone (FQ) and one of the three injectable agents: amikacin (AMK), kanamycin (KAN), or capreomycin (CAP).

Mutations in several genetic loci have been implicated in the development of resistance to SLDs. Resistance to FQs has been reported to be associated with mutations in two regions known as "quinolone resistance-determining regions (QRDRs)", in the *gyrA* (codons 74 to 113) and *gyrB* (recently proposed to span from codon 500 to codon 540) genes, which encode the respective subunits of the DNA topoisomerase gyrase.[1,2] Resistance to KAN, AMK, and CAP is thought to be mediated by mutations in the *rrs* gene, coding for 16S rRNA.[3] Furthermore, resistance to CAP has been shown to be caused by alterations in the *tlyA* gene, coding for 2′-O-methyltransferase, an enzyme that methylates riboses in rRNA.[4] Finally, mutations in a two-gene operon (*ethA*/*ethR*) whose products are involved in the activation of ethionamide (ETH) have been implicated in conferring resistance to this drug.[5]

The purpose of this study was to investigate the prevalence of resistance to SLDs in MDR *Mycobacterium tuberculosis* clinical isolates and to search for genetic determinants of SLD resistance.

Materials and methods

The study included 46 MDR *M. tuberculosis* strains, collected at the National Tuberculosis and Lung Diseases Institute in Warsaw during the third national survey of DR-TB. The isolates were recovered from 46 unrelated pulmonary TB patients (40 men and 6 women; age range, 31—79 years; median age, 50.5 years) from across Poland. These patients represented all bacteriologically-confirmed MDR-TB cases reported in Poland in 2004 [TB cases registered (total), $n = 9493$; TB notification rate, $n = 24.9$/100,000 population]. The detailed sociodemographic and clinical characteristics of the patients whose isolates were used in this study had been presented elsewhere.[6] Primary isolation, cultivation, and species identification were performed with standard mycobacteriological methods.[6] Drug susceptibility testing was carried out using the 1% proportion method on the Löwenstein-Jensen medium. The cutoff drug concentrations were as follows: INH, 0.2 μg/mL; RMP, 40 μg/mL; streptomycin (SM), 4 μg/mL; ethambutol, 2 μg/mL; ofloxacin (OFX), 2 μg/mL; AMK, 4 μg/mL; CAP, 40 μg/mL, and ETH, 40 μg/mL. Genomic DNA was extracted using the cetyltrimethylammonium bromide method, as previously described.[7] The presence of mutations possibly associated with resistance to SLDs was determined by amplifying and sequencing six genetic loci, i.e., two hotspot targeted regions: *gyrA* (codons 17 to 213) and *gyrB* (codons 377 to 612) and the following genes: *rrs* (AMK), *tlyA* (CAP), and *ethA*/*ethR* (ETH). All primers used for amplification were newly designed (Table 1). Amplification reactions were performed according to manufacturer's specific recommendations (TopTaq DNA polymerase, QIAGEN, Hilden, Germany). Purified polymerase chain reaction amplicons were sequenced in both directions using the same primers as for polymerase chain reaction amplification and when needed, additional sequencing primers. All alignments were done against the genome sequence of the *M. tuberculosis* reference strain H37Rv (http://www.ncbi.nlm.nih.gov/genbank/; GenBank accession number: AL123456.3) using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/).

Results

A total of 14 (30.4%; 14/46) strains were resistant to at least one of the four SLDs tested. Nine (19.5%) strains were resistant to either OFX (7; 15.2%) or AMK (2; 4.3%), and thus met the definition of pre-XDR-TB, whereas three (6.5%) strains were resistant to OFX and AMK (2; 4.3%) or CAP (1; 2.2%) simultaneously and were categorized as XDR-TB strains. Four (8.7%) strains, including two pre-XDR strains, were resistant to ETH. The overall frequencies of resistance to SLDs were as follows: 21.7% ($n = 10$) for OFX, 8.7% ($n = 4$) for AMK, 8.7% ($n = 4$) for ETH, 2.2% ($n = 1$) for CAP.

Distribution of mutations and percentage of strains carrying mutations in each of the investigated genes among 46 *M. tuberculosis* strains are shown in Figure 1. Nonsynonymous amino acid changes in the *gyrA*/*gyrB* loci, excluding Ser95Thr (*gyrA*), which is known as natural polymorphism[8,9] occurred in seven (15.2%; 7/46) strains, six of which were resistant to OFX. However, only five (out of 10) OFX-resistant strains contained mutations specific for resistance to this FQ. These were Ala90Val, Asp94Asn, and Asp94Gly in *gyrA* locus (four strains) and Ser447Phe in *gyrB* locus (one strain). None of the four AMK-resistant strains carried mutations in the *rrs* gene. The *rrs* mutations, in two highly mutable regions known as the 530 and 912 loops, were found in seven (15.2%; 7/46) strains susceptible to

Table 1 Oligonucleotide primers used for amplification of the genes analyzed.

Locus	Primer[a]		Annealing temperature (°C)	Amplicon size (bp)
	Designation	Nucleotide sequence (5′→3′)		
gyrA	Fw: gyrAF	ACCGGTTGACATCGAGCAGGAG	59	592
	Rev: gyrAR	CTCTTCGTCGGCGTCGTGATTC		
gyrB	Fw: gyrBF	GCAACACCGAGGTCAAATC	51	710
	Rev: gyrBR	ACCCTTGTACCGCTGAATG		
rrs	Fw: rrsF	TGGCCATGCTCTTGATGC	54	1707
	Rev: rrsR	CGCCCACTACAGACAAGAAC		
	rrs-seqF[b]	TTTACGGCGTGGACTACC		
	rrs-seqR[b]	CAGTAACTGACGCTGAGGAG		
tlyA	Fw: tlyAF	TCTCTGGCCGAACTCGAAGG	58	941
	Rev: tlyAR	GTGTGGACGACCAGCAGAAC		
ethA	Fw: ethAF	GCCTCGACATTACGTTGATAGC	55	1551
	Rev:ethA	CATCATCGTCGTCTGACTATGG		
ethR	Fw: ethRF	AGGCCGTCAACGAGATGTC	54	750
	Rev: ethRR	CATGCCGCTTTGGCACTGAG		

[a] Fw and Rev stand for forward and reverse primers, respectively. All primers used for amplification were also used for sequencing.
[b] Indicates additional intragenic starters used for sequencing.

AMK yet resistant to SM. All MDR-TB strains tested, including one CAP-resistant strain, had *tlyA* G33A (Leu11-Leu) polymorphism. Mutations in the *ethA* gene were detected in one (out of 4) ETH-resistant strain (frameshift mutation 1394delT; this mutation was also found in one ETH-susceptible strain) and 10 ETH-susceptible strains. Only one mutation (T507C; His169His) was found in the *ethR* gene of a single ETH-susceptible strain.

Discussion

This study is the first such extensive analysis on the genetic determinants of SLD resistance in *M. tuberculosis* strains from Poland. It is also among very few reports on the molecular mechanisms of resistance of tubercle bacilli to SLDs. According to a recently published World Health Organization report, a total of 32% of MDR-TB patients excreted bacilli resistant to an FQ, a second-line injectable agent, or both. The average proportion of MDR-TB cases with XDR-TB was calculated at 9.6%.[10] We found these two indicators at 26.1% and 6.5%, respectively.

The prevalence of mutations in *gyrA* and/or *gyrB* loci among FQ-resistant *M. tuberculosis* strains is usually high and may exceed 90%. Point mutations at codons 90, 91, and 94 in *gyrA* are most commonly observed.[11,12] A systematic review by Mauri et al[13] showed mutations in these three codons to account for 54% of FQ-resistant strains. In the present study, the *gyrA* mutations specific for FQ resistance (Ala90Val, Asp94Asn, and Asp94Gly) were demonstrated in 40% (4/10) of OFX-resistant strains. Another OFX-resistant strain had a *gyrB* mutation (Ser447Phe). This mutation, however, had previously been found in OFX-susceptible strains and thus seems to be irrelevant to FQ resistance.[14] Similarly, the Asp89Gly mutation in the *gyrA*, found here in one OFX-resistant and one OFX-susceptible strain, had previously been described in both OFX-resistant and -susceptible strains.[12,15]

The A1401G substitution in the *rrs* gene is considered a highly specific predictor for AMK resistance, having been observed in close to 80% of AMK-resistant strains and never in AMK-susceptible strains.[16] Surprisingly, this mutation was absent in our four AMK-resistant strains. The only *rrs* mutations detected were within the 530 and 912 loops and involved SM-resistant strains. The genetic determinants of resistance to SM, within the sample studied, were investigated in our previous report.[17] These mutations were also described in other studies and occurred in *M. tuberculosis* strains resistant to SM, with or without additional resistance to other aminoglycosides, including AMK. This observation supports previous findings that mutations in the 530 and 912 regions of the *rrs* gene are associated with resistance to SM but not AMK or other aminoglycosides.[17–19]

The only commercially available molecular diagnostic assay to detect resistance of tubercle bacilli to SLDs, GenoTypeMTBDR*sl* (Hain Lifescience, Nehren, Germany), harbors mutations at codons 90, 91, and 94 of the *gyrA* gene and mutations at 1401, 1402, and 1484 nucleotide positions of the *rrs* gene as markers predictive of resistance to FQs and aminoglycosides, accordingly. Based on the results presented here, this test would fail to disclose 60% of strains resistant to OFX and all strains resistant to AMK.

The association of mutations in the *tlyA* gene with resistance to CAP was first inferred from an *in vitro* study.[4] Generally, CAP-resistance-related *tlyA* mutations are rare (found in up to 3% of CAP-resistant strains). A GT insertion at position 755 has been reported most frequently and only in CAP-resistant strains.[16] Here, the only *tlyA* mutation, found across the whole sample, was a synonymous transition G33A (Leu11Leu). This mutation has recently been described by Engström et al[19] in the pan-susceptible reference strains H37Rv and H37Ra, and the *in vitro* selected CAP, AMK, and KAN-resistant mutants derived from them. Apart from the *tlyA* gene, the only other locus evaluated in the context of CAP-resistance has been the promoter region of the *eis* gene, coding for an aminoglycoside acetyltransferase. However, since mutations at

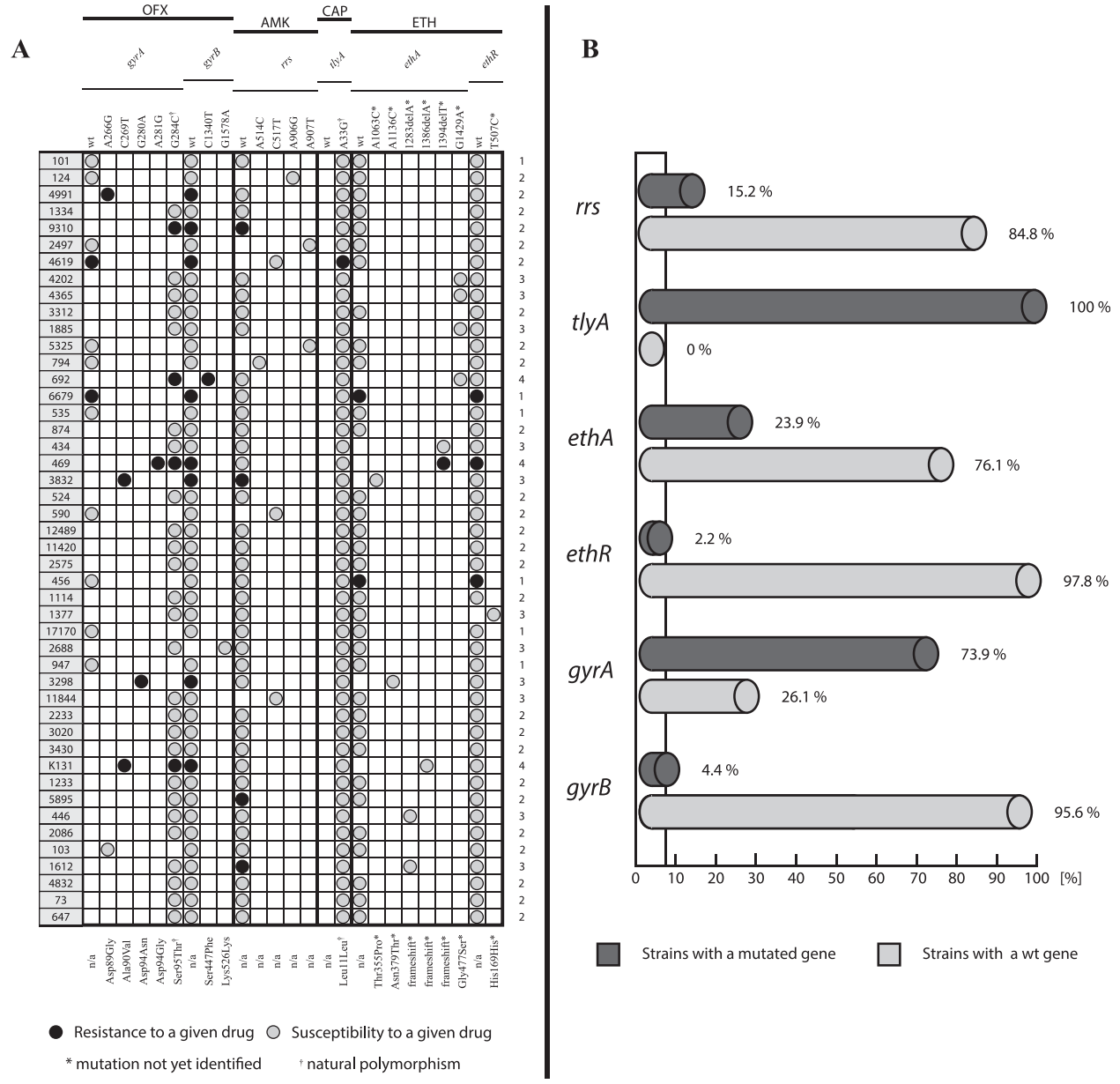

Figure 1. (A) Distribution of mutations in the investigated genes among 46 *Mycobacterium tuberculosis* strains tested. The total number of gene mutations in particular strains is given on the right side of the chart; (B) graph illustrating the percentage of strains carrying mutations in the investigated genes versus the percentage of strains with wt alleles of these genes.

this locus have largely been shown to be nonspecific for CAP-resistant strains,[16,20] we decided not to perform the sequence analysis of the *eis* promoter in our study. It seems that genetic markers of CAP resistance have not yet been uncovered.

While the ability of EthA and EthR to activate ETH has been described in previous studies, limited data exist on the presence of *ethA* and *ethR* mutations in *M. tuberculosis* clinical isolates.[21] We found six mutation types in the *ethA* gene in 23.9% of the strains under the study and none of them had previously been described. This is in line with a high degree of genetic diversity within the *ethA* gene in *M. tuberculosis*, as evidenced in past studies. For example, Leung et al[22] detected 52 different mutation types among 63 *M. tuberculosis* strains (52 ETH-resistant and 11 ETH-susceptible) with an altered *ethA* gene. In this study, only one out of four ETH-resistant strains contained a mutation in the *ethA*/*ethR* locus. Previously, the prevalence of mutations in the *ethA*/*ethR* operon has been estimated to range from 37% to 100%.[5,23,24] According to the literature, the only mutations in the *ethR* gene consistently related to ETH-resistance are Ala95Thr and Phe110Leu.[5] These were both undetected in our sample.

Conclusion

In conclusion, the prevalence of mutations in tested loci was similar to those described in the literature. An exception was the lack of A1401G substitution in the *rrs* gene,

probably due to a limited number of AMK-resistant strains in the sample studied. Apart from two *gyrA* mutations (at codons 90 and 94), none of the other changes detected in all six genes tested could be associated with resistance to SLDs. This casts doubt over the usefulness of sequence analyses of tested genes (except QRDR of *gyrA*) for the prediction of SLD resistance patterns and highlights the need for searching other genetic loci for detection of mutations conferring resistance to SLDs.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: KJ872562 KJ872567 for the *gyrA* mutants; KJ921741 and KJ921742 — *gyrB*; KF796660 —KF796662 and KF796665 — *rrs*; KJ872561 — *tlyA*; KJ872554-KJ872559 — *ethA*; KJ872560 — *ethR*.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Part of the results of this study were presented as a poster (P0936) at the 24th European Congress of Clinical Microbiology and Infectious Diseases in Barcelona, Spain, 10—13 May 2014. This study was supported by the «Iuventus Plus» grant from the Polish Ministry of Science and Higher Education (research contract no. IP2011018771).

References

1. Devasia R, Blackmanb A, Edenc S, Li H, Maruri F, Shintani A, et al. High proportion of fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates with novel gyrase polymorphisms and a *gyrA* region associated with fluoroquinolone susceptibility. *J Clin Microbiol* 2012;**50**:1390—6.
2. Pantel A, Petrella S, Veziris N, Brossier F, Bastian S, Jarlier V, et al. Extending the definition of the *gyrB* Quinolone Resistance-Determining Region in *Mycobacterium tuberculosis* DNA gyrase for assessing fluoroquinolone resistance in *M. tuberculosis*. *Antimicrob Agents Chemother* 2012;**56**:1990—6.
3. Du Q, Dai G, Long Q, Yu X, Dong L, Huang H, et al. *Mycobacterium tuberculosis rrs* A1401G mutation correlates with high-level resistance to kanamycin, amikacin, and capreomycin in clinical isolates from mainland China. *Diagn Microbiol Infect Dis* 2013;**77**:138—42.
4. Maus CE, Plikaytis BB, Shinnick TM. Mutation of *tlyA* confers capreomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2005;**49**:571—7.
5. Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W. Molecular investigation of resistance to the antituberculous drug ethionamide in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2011;**55**:355—60.
6. Jagielski T, Augustynowicz-Kopeć E, Zozio T, Rastogi N, Zwolska Z. Spoligotype-based comparative population structure analysis of multidrug-resistant and isoniazid-monoresistant *Mycobacterium tuberculosis* complex clinical isolates in Poland. *J Clin Microbiol* 2010;**48**:3899—909.
7. van Embden JDA, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 1993;**31**:406—9.
8. Lau RWT, Ho P-L, Kao RYT, Yew WW, Lau TC, Cheng VC, et al. Molecular characterization of fluoroquinolone resistance in *Mycobacterium tuberculosis*: functional analysis of *gyrA* mutation at position 74. *Antimicrob Agents Chemother* 2011;**55**:608—14.
9. Ali A, Hasan R, Jabeen K, Jabeen N, Qadeer E, Hasan Z. Characterization of mutations conferring extensive drug resistance to *Mycobacterium tuberculosis* isolates in Pakistan. *Antimicrob Agents Chemother* 2011;**55**:5654—9.
10. World Health Organization. *Global tuberculosis control: surveillance, planning, financing*. Geneva, Switzerland: WHO; 2013.
11. Perdigão J, Macedo R, Malaquias A, Ferreira A, Brum L, Portugal I. Genetic analysis of extensively drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal. *J Antimicrob Chemother* 2010;**65**:224—7.
12. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et al. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2009;**55**:2032—41.
13. Maruri F, Sterling TR, Kaiga AW, Blackman A, van der Heijden YF, Mayer C, et al. A systematic review of gyrase mutations associated with fluoroquinolone-resistant *Mycobacterium tuberculosis* and a proposed gyrase numbering system. *J Antimicrob Chemother* 2012;**67**:819—31.
14. Sirgel FA, Warren RM, Streicher EM, Victor TC, van Helden PD, Böttger EC. *gyrA* mutations and phenotypic susceptibility levels to ofloxacin and moxifloxacin in clinical isolates of *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2012;**67**:1088—93.
15. Duong DA, Nguyen TH, Nguyen TN, Dai VH, Dang TM, Vo SK, et al. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with high-level fluoroquinolone resistance in Vietnam. *Antimicrob Agents Chemother* 2009;**53**:4835—9.
16. Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell TC. Evaluation of genetic mutations associated with *Mycobacterium tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review. *PLoS One* 2012;**7**:e3327. http://dx.doi.org/10.1371/journal.pone.0033275.
17. Jagielski T, Ignatowska H, Bakuła Z, Dziewit L, Napiórkowska A, Augustynowicz-Kopeć E, et al. Screening for streptomycin resistance-conferring mutations in *Mycobacterium tuberculosis* clinical isolates from Poland. *PLoS One* 2014;**9**:e100078. http://dx.doi.org/10.1371/journal.pone.0100078.
18. Jugheli L, Bzekalava N, de Rijk P, Fissette K, Portaels F, Rigouts L. High level of cross-resistance between kanamycin, amikacin, and capreomycin among *Mycobacterium tuberculosis* isolates from Georgia and a close relation with mutations in the *rrs* gene. *Antimicrob Agents Chemother* 2009;**53**:5064—8.
19. Engström A, Perskvist N, Werngren J, Hoffner SE, Juréen P. Comparison of clinical isolates and in vitro selected mutants reveals that *tlyA* is not a sensitive genetic marker for capreomycin resistance in *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2011;**66**:1246—54.
20. Rodwell TC, Valafar F, Douglas J, Qian L, Garfein RS, Chawla A, et al. Predicting extensively drug-resistant *Mycobacterium tuberculosis* phenotypes with genetic mutations. *J Clin Microbiol* 2014;**52**:781—9.

21. Dover LG, Corsino PE, Daniels IR, Cocklin SL, Tatituri V, Besra GS, et al. Crystal structure of the TetR/CamR family repressor *Mycobacterium tuberculosis* EthR implicated in ethionamide resistance. *J Mol Biol* 2004;**340**:1095—105.
22. Leung KL, Yip CW, Yeung YL, Wong KL, Chan WY, Chan MY, et al. Usefulness of resistant gene markers for predicting treatment outcome on second-line anti-tuberculosis drugs. *J Appl Microbiol* 2010;**109**:2087—94.
23. Morlock GP, Metchock B, Sikes D, Crawford JT, Cooksey RC. *ethA*, *inhA*, and *katG* loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates. *Antimicrob Agents Chemother* 2003;**47**:3799—805.
24. De Barber AE, Mdluli K, Bosman M, Bekker LG, Barry CE. Ethionamide activation and sensitivity in multidrug-resistant *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 2000;**97**:9677—82.