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Biological cost in *Mycobacterium tuberculosis* with mutations in the *rpsL*, *rrs*, *rpoB*, and *katG* genes

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SUMMARY

When bacteria develop drug-resistant mutations, there is often an associated biological cost; however, some strains can exhibit low- or no-cost mutations. In the present study, a quantitative resazurin reduction assay was used to measure the biological cost of *Mycobacterium tuberculosis* isolates that contained different mutations in the *rpsL*, *rrs*, *rpoB*, and *katG* genes, and showed different resistance profiles. Biological costs were determined by comparing the growth curves of drug-resistant isolates with drug-susceptible strains. Some strains, such as those with *rpoB* mutations other than S531L and strains with mutations in all of the studied genes, grew more slowly than did drug-susceptible strains. However, some strains grew more quickly than drug-susceptible strains, such as those that had only the *rpsL* K43R mutation. Strains with the mutation *katG* S315T presented heterogeneous biological costs. When analyzed individually, strains with the mutations *rpsL*43/*katG*315, *rpoB*531, and *rpoB*531/*katG*315 grew faster than drug-susceptible strains. The results suggest that some strains with the most common mutations correlated to a high resistance toward streptomycin, isoniazid and rifampicin can grow as well as or better than susceptible strains.

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1. Introduction

Tuberculosis (TB) is considered to be multidrug resistant (MDR) when its causal agent, *Mycobacterium tuberculosis*, is resistant to at least rifampicin (RIF) and isoniazid (INH), which are the main first-line drugs used to treat TB. Resistance to these drugs requires the administration of less effective, more toxic, and more costly drugs.¹ The molecular mechanisms of resistance have been determined for many antituberculosis agents. It has been shown that *M. tuberculosis*

isolates essentially become resistant through mutations that occur spontaneously at low frequency in chromosomal genes. In clinical practice, drug resistance occurs as a result of inadequate therapeutic regimens or patient failure to comply with treatment.²

INH is one of the main anti-TB drugs; mutations in the *katG* gene are related to INH resistance, with *katG* S315T mutation being the most common in INH-resistant strains.^{2,3} Many studies have shown that RIF-resistance is due to mutations mostly within a defined region of 81 bp of the *rpoB* gene, which encodes the β-subunit of the RNA polymerase. Mutations in this region of the gene have been found in approximately 96% of the RIF-resistant *M. tuberculosis* strains that were recovered globally.^{4,5} Another first-line drug used in the treatment of TB is streptomycin (STR). Many STR-resistant strains have mutations in the *rpsL* and *rrs* genes that encode the ribosomal protein S12 and 16S ribosomal RNA, respectively.⁶

Acquiring drug resistance in bacteria often carries a biological cost because antibiotics generally target essential, highly conserved genes.⁷ Although drug-resistant strains often suffer an initial reduction in fitness, they continue to evolve by acquiring one or

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more secondary-site mutations that can improve or even restore the fitness over time.⁷ Furthermore, although the acquisition of drug resistance determinants is often associated with a loss in fitness, some strains with low- or no-cost mutations have been reported. For example, the mutation in codon 43 of the *rpsL* gene,⁸ the mutation *katG* S315T,⁹ and the mutation *rpoB* S531L were described as having a relative fitness greater than or equal to 1.0, which is considered to be the fitness of the susceptible *M. tuberculosis* strain.^{10,11} Some mutations have implications in virulence. For example, the *katG* mutation S315T has a high degree of INH resistance, retains enzyme activity⁹ and is the only *katG* mutation associated with successful transmission.¹²

The laboratory approaches for estimating the biological cost of drug-resistant *M. tuberculosis* include measuring the growth rate^{13–15} and infectivity in animal models.¹⁶ The fitness cost of drug resistance is usually measured relative to the corresponding wild-type strain in the absence of antibiotics, where the resistant strain has to compete with its susceptible counterparts.^{10,11,14,17}

As mentioned above, most studies generally consider only one mutation in one gene related to drug resistance, and few studies have examined the possible variable effects of cumulative mutations in different genes at the same time on *M. tuberculosis* fitness. The aim of this study was to assess the biological cost of *M. tuberculosis* by comparing the growth curves, which were measured by spectrophotometric quantification of resazurin reduction¹⁸ in MDR and susceptible strains.

2. Material and methods

2.1. Strains

Twenty-one MDR (nine of which were also STR-resistant) *M. tuberculosis* clinical isolates with different mutations in the genes *rpsL*, *rrs*, *rpoB*, and *katG* and three susceptible clinical isolates obtained from LACEN-RS – Rio Grande do Sul Central State Laboratory Collection were selected for this study. The strains were selected based upon their resistance profiles. The *M. tuberculosis* strains with their respective resistance profile and gene mutations are shown in Table 1.

2.2. MIC determination

Resazurin microtiter assay (REMA) was used for MIC determination.¹⁹ Microplates were filled with 7H9 plus 10% oleic acid-albumin-dextrose-catalase (7H9-OADC) medium (Becton Dickinson, USA). Serial 1:2 dilutions of STR (125 µg/ml to 0.25 µg/ml), RIF (16 µg/ml to 0.03 µg/ml) and INH (12.8 µg/ml to 0.01 µg/ml) were performed. The MIC used to define breakpoint resistance was 1 µg/ml for STR and 0.25 µg/ml for RIF and INH.²⁰ MDR strains were tested with three second-line drugs, including ofloxacin (OFL, 8–0.25 µg/ml), kanamycin (KAN, 20–0.62 µg/ml), and capreomycin (CAP, 10–0.3 µg/ml), with breakpoints of 2 µg/ml, 2.5 µg/ml and 2.5 µg/ml, respectively.²¹

2.3. DNA extraction and sequencing

DNA was isolated from mycobacterial cultures by the lysozyme/proteinase K cetyltrimethylammonium bromide procedure.²² DNA fragments from *M. tuberculosis* *rpsL* and *rrs* genes sized 306 and 238 bp, respectively, were amplified as described by Tracevska et al.²³ using the respective primers SM1-CCAACCATCCAGCAGCTGGT and SM2-ATCCAGCGAACCGCGGATGA and SM3-GATGACGGCCTGGGTGT and SM4-TCTAGCTGCCGTATGCC. The 157 bp region coding the hot spot fragment of the *rpoB* gene was amplified as described in Telenti et al.⁴ using the primers RF1-GGTCGCCGCGATCAAGGAGT and

Table 1

Resistance profile to RIF, INH and STR, mutations in the *rpsL*, *rrs*, *rpoB* and *katG* genes and the spoligotyping lineage of the strains studied.

Strain no.	Resistance*	Mutation†	Lineage
500270	Susceptible	—	LAM1
621042	Susceptible	—	T3
621113	Susceptible	—	LAM2
621047	MDR + S + K	<i>rpsL</i> 81/ <i>rpoB</i> 526Y/ <i>katG</i> 315	H3
621052	MDR + S	<i>rpsL</i> 43/ <i>rpoB</i> 531/ <i>katG</i> 315	T5_MAD2
621172	MDR + S + K	<i>rpsL</i> 43/ <i>rpoB</i> 526Y/ <i>katG</i> 315	T5_MAD2
621173	MDR + S + K	<i>rpsL</i> 43/ <i>rpoB</i> 531/ <i>katG</i> 315	LAM6
500281	MDR + S	<i>rrs</i> 513C-T/ <i>rpoB</i> 531/ <i>katG</i> 315	LAM5
500425	MDR + S	<i>rrs</i> 513C-T/ <i>rpoB</i> 531/ <i>katG</i> 315	H1
600726	MDR + S	<i>rpoB</i> 531/ <i>katG</i> 315	LAM5
500294	MDR + S	<i>rpsL</i> 43/ <i>katG</i> 315	LAM
500201	MDR + S	<i>rpsL</i> 43/ <i>katG</i> 315	T5_MAD2
600007	MDR	<i>rpoB</i> 531/ <i>katG</i> 315	LAM9
621044	MDR	<i>rpoB</i> 531/ <i>katG</i> 315	LAM5
621049	MDR	<i>rpoB</i> 531/ <i>katG</i> 315	LAM5
621164	MDR	<i>rpoB</i> 531/ <i>katG</i> 315	LAM5
621230	MDR	<i>rpoB</i> 531/ <i>katG</i> 315	LAM5
621241	MDR	<i>rpoB</i> 531/ <i>katG</i> 315	LAM5
621242	MDR	<i>rpoB</i> 531/ <i>katG</i> 315	LAM5
6001061	MDR	<i>rpoB</i> 531/ <i>katG</i> 315	LAM5
621087	MDR	<i>rpoB</i> 516/ <i>katG</i> 315	LAM
621257	MDR	<i>rpoB</i> 516/ <i>katG</i> 315	LAM
621192	MDR	<i>rpoB</i> 526P/ <i>katG</i> 315	non-LAM
600599	MDR	<i>rpoB</i> 531	LAM9

* MDR – strain phenotypic resistant to INH and RIF; MDR + S – strain phenotypic resistant to RIF, INH and STR; MDR + S + K – strain phenotypic resistant to RIF, INH, STR and KAN.

† No mutation; *rpoB* – TCG → TTG, S531L; CAC → TAC, H526Y; CAC → CCC, H526P and ins 516 CCAGAACAAACCC; *rpsL* – AAG → AGG, K43R and CTG → TTG, L81L; *katG* – AGC → ACC, S315T.

RF2-TGCACGTCGGGACCTCCA. The 232 bp fragment of the *katG* gene was amplified as described in Dalla Costa et al.³ using the primers KatG1-CATGAACGACGTCGAAACAG and KatG2-CGAGGAAACTGTTG TCCCCAT. Sequencing was performed using an ABI Prism 3100 DNA sequencer (Applied Biosystems). Nucleotide sequences were then analyzed using the programs PREGAP and GAP4 of the STADEN software package, ver. 10.0. Nucleotide sequences with Phred values > 20 were considered for analysis.

2.4. Genotyping

Sporolotyping was performed using a commercial kit (Isogen Biosciences B.V., The Netherlands) according to the manufacturer's instructions. The Shared International Types (SITs) and families were identified according to the SpolDB4 database.²⁴ The strains were further characterized by a LAM-specific PCR to classify strains that could not be assigned to an internationally recognized genotype lineage. In this assay, PCR primers were used to identify the presence of an IS6110 insertion, which is unique to all members of the LAM lineage.²⁵

2.5. Biological cost measurement

The biological cost of *M. tuberculosis* was determined by calculating growth curves for each strain. The time of growth for each strain was measured with a resazurin reduction assay that quantified mycobacterial metabolism spectrophotometrically, as described in von Groll et al. 2010, with modifications.¹⁸ Briefly, subcultures of strains were made in Löwenstein–Jensen slants 21 days before the test. The inoculums were prepared as follows: a suspension of each strain was diluted in autoclaved water to match the McFarland No. 1 standard and was then diluted 1:20 in 7H9-OADC medium. Wells in a 96-well plate were filled with 100 µl 7H9-OADC medium in the test wells and the negative control

(resazurin control), then 100 µl inoculum from each strain were added in triplicate. The plate was then sealed and incubated at 37 °C. After 48 h, 30 µl 0.02% resazurin was added, and the plate was returned to 37 °C and the readings were measured every 12 h for 7 days at 620 nm using a plate reader (TECAN Spectrum Classic). All tests were performed during the same week and with the same prepared reagents. The growth curve was then calculated from the mean optical density (OD) of the triplicate culture, from which the mean OD of the negative controls was subtracted. Samples were compared as a function of time (h). The biological cost of each strain was estimated for two parameters¹⁸: the length of the lag phase, which was the time for which samples took to reach an OD of 0.2 from the start of incubation, and the growth index, which was the time for each strain to reach an OD of 0.4 from an OD of 0.2. The growth index was calculated with the assumption that all strains were in the logarithmic growth phase between the two OD values.¹⁸

3. Results

3.1. Molecular characterization

Of the 24 strains, three strains were susceptible and had no mutations in the sequenced genes. Of the other 21 strains, nine were phenotypically resistant to INH, RIF and STR (MDR + S); four had mutations in three genes (*rpsL81/rpoB526Y/katG315*, *rpsL43/rpoB531/katG315*, *rpsL43/rpoB526Y/katG315*, *rpsL43/rpoB531/katG315*); two had mutations in three other genes (*rrs513C-T/rpoB531/katG315*); two had mutations in the *rpsL* and *katG* genes (*rpsL43/katG315*); and one had mutations in the *rpoB* and *katG* genes (*rpoB531/katG315*). From these strains, three were found to have additional resistance to KAN (Table 1 and Supplementary Material). Twelve strains were

phenotypically resistant to INH and RIF (MDR) and are described as follows: eight had two mutations (*rpoB531/katG315*), two had another two-gene mutation (*rpoBins516/katG315*), one had a third two-gene mutation (*rpoB526P/katG315*) and one had a single-gene mutation (*rpoB531*) (Table 1).

Of the strains studied, 17 were classified as belonging to the Latin American-Mediterranean lineage (sub lineages: three LAM, one LAM1, one LAM2, nine LAM5, one LAM6, and two LAM9), and seven were classified as non-LAM (sub lineages: three T5-MAD2, one Haarlem1, one Haarlem3, one T3, one non-LAM) (Table 1).

3.2. Biological cost

The length of the lag phase and the growth index for all strains are shown in Figure 1 and the Supplementary Material. The growth curves of strains were grouped averaged and are compared in Table 2. When comparing the length of the lag phase and growth index, susceptible strains had the fastest growth, followed by the MDR strains and then the MDR + S strains.

Strains were grouped according to the mutations found in the genes *rpsL*, *rrs* and *rpoB*; however, the *katG* S315T mutation was not utilized for grouping because almost all strains contained this mutation (20/21). The lag phase was shorter in the wild-type strains, followed sequentially by the strains with the *rpsL* K43R mutation, strains with the *rpoB* S531L mutation, strains with *rpoB* mutations excluding S531L, strains with mutations in both *rpsL* and *rpoB*, and lastly, strains with mutations in both *rrs* and *rpoB*. The growth index showed that the group with the fastest growth contained strains with mutations in *rpsL* K43R, followed by *rpoB* S531L mutated strains, wild-type strains, strains with mutations in *rpsL* and *rpoB*, strains with mutations in *rrs* and *rpoB*, and lastly, the strains mutated in *rpoB* except in S531L (Table 2).

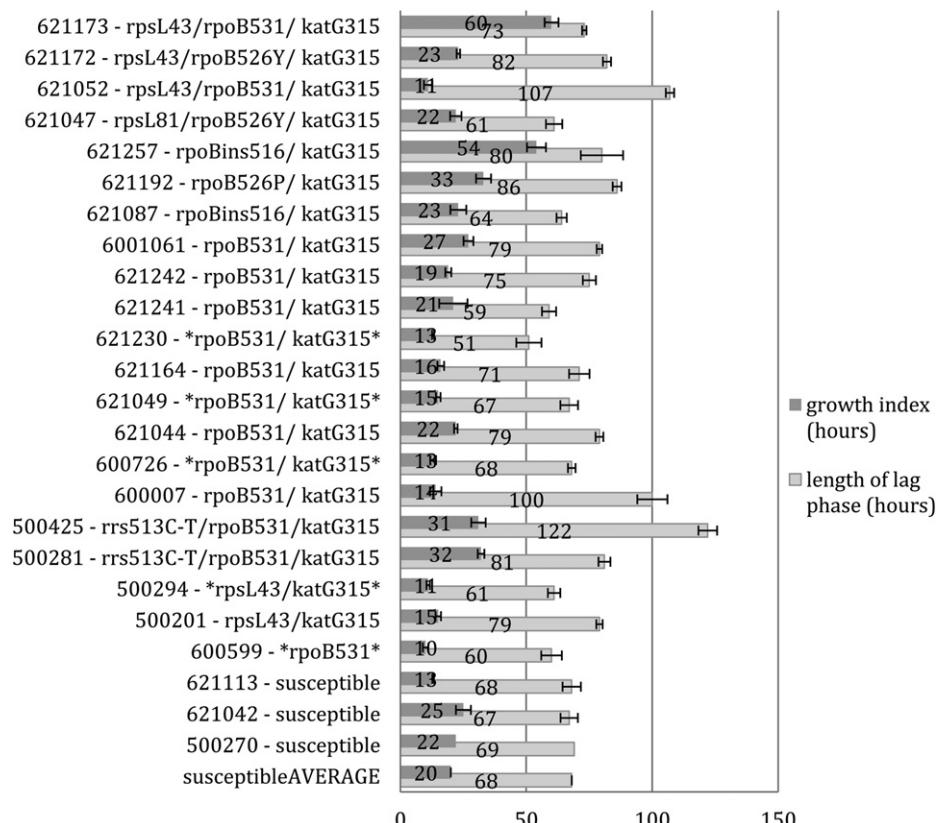


Figure 1. The growth index and the length of the lag phase for each strain, with standard deviation of triplicates (hours). *Strains that have the length of the lag phase and the growth index values lower than the average for the susceptible strains.

Table 2
Comparisons of different groups of strains.

Comparison	No.	Length of lag phase (h)		Growth index (h)	
		Average	St. deviation	Average	St. Deviation
Susceptible	3	68.0	1.0	20.0	6.2
MDR + S	9	81.6	20.6	24.2	15.6
MDR	12	72.6	13.5	22.3	11.9
WT	3	68.0	1.0	20.0	6.2
rpsL43	2	70.0	12.7	13.0	2.8
rpoB531	10	70.9	13.6	17.0	5.2
rpoB other	3	76.7	11.4	36.7	15.8
rpsL and rpoB	4	80.8	19.5	29.0	21.4
rrs and rpoB	2	101.5	29.0	31.5	0.7
non-LAM	7	86.3	21.6	22.9	7.9
LAM	17	70.9	11.2	22.6	14.3

MDR – strain phenotypic resistant to INH and RIF; MDR + S – strain phenotypic resistant to RIF, INH and STR; WT – wild type, strain without mutations in the studied genes; h – hours.

Comparison of the average growth of susceptible strains with individual mutated strains shows that some individual mutated strains had the length of the lag phase and the growth index values lower than the mean of the susceptible strains. These resistant strains grew faster than the mean of the susceptible strains that were measured. This was the case of strains with mutations in *rpsL43/katG315* (500294), *rpoB531* (600599), *rpoB531/katG315* (600726, 621049, 621164), as shown in Figure 1.

Comparison of the biological cost parameters between LAM and non-LAM groups showed a shorter lag phase for LAM strains and a similar growth index for both groups, including all strains (Table 2), when only the resistant ones were analyzed the results were kept (data not shown). No statistical analysis was done because of the small sample number; however, the results suggest biological significance.

4. Discussion

Dissemination of antimicrobial resistant strains occurs even though drug resistance can have a biological cost for the microorganism. The fitness cost of resistance is among the most important factors determining both the rate and the extent of resistance emergence.²⁶

In this study, MDR *M. tuberculosis* strains, including STR-resistance and the most common STR, RIF and INH drug resistance-conferring mutations, were selected. These mutations included the most frequently isolated strains that have been published in several studies, including the mutations *rpsL* K43R,^{6,27} *rpoB* S531L^{2,28} and *katG* S315T.^{3,28} We also find three strains that were resistant to KAN, considered to be pre-XDR strains, defined as MDR resistant to kanamycin but susceptible to fluoroquinolones²⁹ (Table 1 and Supplementary Material). In some strains, the results of phenotypic antimicrobial susceptibility tests and genotypic molecular analysis are inconsistent (Table 1 and Supplementary Material). In these strains, heteroresistance³⁰ or mutations in genes related with low-level of resistance could be occurring.

In our study, we compared the length of the lag phase and growth index of 21 MDR *M. tuberculosis* and three drug-susceptible strains. The lag phase is the period required for the adaptation of bacteria to their new environment. During this time, enzymes are actively produced to metabolize novel nutrients. The growth index is associated with the reproductive capacity of the bacteria. Strains that are more metabolically active reproduce more rapidly. We analyzed the growth curves using these two parameters for all grouped strains.

Biological cost was observed in several strains when considering both lag phase and growth index. MDR + S strains grew slower than others; strains with mutations in *rpoB* not including those on codon

531 and strains with mutations in *rpoB*, *rpsL*, *rrs* and *katG* grew at the same rate. Previous studies on MDR strain fitness have demonstrated that clinical isolates can pay a physiological cost for the development of drug-resistance.³¹ Studies considering the *rpoB* gene have found that strains with mutations other than *rpoB* S531L have a biological cost.^{10,17}

In this study, we found that some strains grew better than the susceptible strains when considering the log phase (growth index), particularly those with mutations in *rpsL* K43R or *rpoB* S531L. The previously mentioned *rpoB* studies found that strains with the mutation *rpoB* S531L confer a relatively low fitness cost when compared to strains with other *rpoB* mutations.^{10,11,17}

This study found that MDR + S strains ($n = 9$) grew slower than susceptible strains. However, MDR + S strains with the mutation *rpsL* K43R ($n = 2$, without mutation in the hot spot *rpoB* region of 81 bp) had the opposite behavior. This same mutation is an apparent 'no-cost' mutation in *Salmonella enterica* subsp. *enterica* serovar Typhimurium and in *Escherichia coli*.^{32,33} This substitution is frequently found in STR resistant clinical isolates of *M. tuberculosis*.³⁴

The *katG* S315T mutated strains presented heterogeneous fitness, which was likely due to the presence of other confounding mutations in other loci. When the lag phase and growth index of individually mutated isolates were compared with the mean of susceptible strains, it was found that isolates with the mutations in *katG* S315T and other genes grew faster than the susceptible strains (1 strain: *rpsL43/katG315* and 3 strains: *rpoB531/katG315*). It has been shown that strains with the *katG* S315T mutation retain virulence in a mouse model of TB infection and produce functional catalase-peroxidase with enzymatic activities that are comparable to wild-type. These strains have minimal reduction in fitness⁹ and cause secondary cases of TB as frequently as INH-susceptible strains.³⁵

The three strains with additional resistance to KAN do not differentiate in fitness from the other strains (data not shown). We found that when more mutations are added to the analysis, strains grow more slowly, and standard deviations increase (Figure 1). The results of other studies in strains resistant to INH, STR or RIF suggested that in clinical settings, there is a strong selection pressure for drug resistance-conferring mutations that cause minimal fitness defects.^{7,36}

The strains were from different lineages, with 71% of the strains being classified as LAM lineage and 29% as other lineages, including T and Haarlem. These are the three main lineages described in South America. LAM is the most common lineage and responsible for more than 50% of the TB cases in Latin-America.^{3,24} The variable genetic background of strains belonging to different strain lineages could play a role in the fitness of *M. tuberculosis*.⁷ In this study, strains from different lineages were analyzed. LAM strains showed a shorter length of the lag phase but reached the OD of 0.4 in the same time as did non-LAM strains. In a study by von Groll et al. examining Brazilian strains,¹⁵ LAM strains were found to have a significantly faster growth index than non-LAM strains, however, LAM strains were drug-susceptible, and only one (1/40) was MDR.

A limitation of our study is that the strains are clinical isolates from TB patients, and the genetic backgrounds of the organisms are different and unknown; furthermore, the number of strains examined was limited. The results must therefore be interpreted with caution. Moreover, the mutations responsible for drug resistance development that decreases growth and initial biological cost can be compensated by subsequent mutations that restore bacteria's capacity for growth.²⁶

Several studies have contributed to our knowledge of the relationship of mutations that determine MDR resistance in *M. tuberculosis*. Otherwise, studies where profiles of resistance and mutations have been associated with biological cost are less frequent. Here we have shown that some strains with the most frequent mutations and high level SMR, INH and RIF resistance can

grow as much as or even better than the average for the susceptible strains. The important lesson that these clinical and molecular studies teach us is that some drug-resistant organisms can spread over time due to a natural selection of strains without biological cost. To prevent an epidemic of MDR-TB, we must take care to ensure that all patients are correctly diagnosed and treated avoiding the occurrence and transmission of resistant strains in the community.³⁷

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.tube.2012.11.004>.

References

- Almeida Da Silva PE, Palomino JC. Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. *J Antimicrob Chemother* 2011;66:1417–30.
- Zhang Y, Yew WW. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* 2009;13:1320–30.
- Dalla Costa ER, Ribeiro MO, Silva MS, Arnold LS, Rostirolla DC, Cafrune PI, Espinoza RC, Palaci M, Telles MA, Ritacco V, Suffys PN, Lopes ML, Campelo CL, Miranda SS, Kremer K, da Silva PE, Fonseca Lde S, Ho JL, Kritski AL, Rossetti. Correlations of mutations in *katG*, *oxyR-ahpC* and *inhA* genes and in vitro susceptibility in *Mycobacterium tuberculosis* clinical strains segregated by spoligotype families from tuberculosis prevalent countries in South America. *BMC Microbiol* 2009;19:39.
- Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T. Detection of rifampicin resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 1993;341:647–50.
- Aslan G, Tezcan S, Serin MS, Emekdas G. Genotypic analysis of isoniazid and rifampin resistance in drug-resistant clinical *Mycobacterium tuberculosis* complex isolates in Southern Turkey. *Jpn J Infect Dis* 2008;61:255–60.
- Finken M, Krischner P, Meier A, Wrede A, Böttger EC. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Mol Microbiol* 1993;9:1239–46.
- Borrel S, Gagneux S. Infectiousness, reproductive fitness and evolution of drug-resistant *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* 2009;13:1456–66.
- Böttger EC, Springer B. Tuberculosis: drug resistance, fitness, and strategies for global control. *Eur J Pediatr* 2008;167:141–8.
- Pym AS, Saint-Joanis B, Cole ST. Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans. *Infect Immun* 2002;70:4955–60.
- Billington OJ, McHugh TD, Gillespie SH. Physiological cost of rifampin resistance induced *in vitro* in *Mycobacterium tuberculosis*. *Antimicrobial Agents Chemother* 1999;43:1866–9.
- Mariam DH, Mengistu Y, Hoffner SE, Andersson DI. Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*. *Antimicrobial Agents Chemother* 2004;48:1289–94.
- Gagneux S, Burgos MV, DeRiemer K, Enciso A, Muñoz S, Hopewell PC, Small PM, Pym AS. Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*. *PLoS Pathog* 2006;2:e61.
- Toungoussova OS, Caugant DA, Sandven P, Mariandyshev AO, Bjune G. Impact of drug resistance on fitness of *Mycobacterium tuberculosis* strains of the W-Beijing genotype. *FEMS Immunol Med Microbiol* 2004;42:281–90.
- von Groll A, Martin A, Stehr M, Singh M, Portaels F, da Silva PE, Palomino JC. Fitness of *Mycobacterium tuberculosis* strains of the W-Beijing and Non-W-Beijing genotype. *PLoS One* 2010;5:e10191.
- von Groll A, Martin A, Felix C, Prata PF, Honscha G, Portaels F, Vandame P, da Silva PE, Palomino JC. Fitness study of the RD¹⁰ lineage and Latin American Mediterranean family of *Mycobacterium tuberculosis* in the city of Rio Grande, Brazil. *FEMS Immunol Med Microbiol* 2010;58:119–27.
- Ordway DJ, Sonnenberg MG, Donahue SA, Belisle JT, Orme IM. Drug-resistant strains of *Mycobacterium tuberculosis* exhibit a range of virulence for mice. *Infect Immun* 1995;63:741–3.
- Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohanna B. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science* 2006;312:1944–6.
- von Groll A, Martin A, Portaels F, da Silva PEA, Palomino JC. Growth kinetics of *Mycobacterium tuberculosis* measured by quantitative resazurin reduction assay: a tool for fitness studies. *Braz J Microbiol* 2010;41:300–3.
- Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrobial Agents Chemother* 2002;46:2720–2.
- Montoro E, Lemus D, Echemendia M, Martin A, Portaels F, Palomino JC. Comparative evaluation of the nitrate reduction assay, the MTT test, and the resazurin microtiter assay for drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2005;55:500–5.
- Martin A, Camacho M, Portaels F, Palomino JC. Resazurin microtiter assay plate testing of *Mycobacterium tuberculosis* susceptibilities to second-line drugs: rapid, simple, and inexpensive method. *Antimicrobial Agents Chemother* 2003;47:3616–9.
- van Soolingen D, de Hess PE, Hermans PW, van Embden JD. DNA fingerprinting of *Mycobacterium tuberculosis*. *Methods Enzymol* 1994;235:196–205.
- Tracevska T, Jansone I, Nodieva A, Marga O, Skenderi G, Baumanis V. Characterisation of *rpsL*, *rrs* and *embB* mutations associated with streptomycin and ethambutol resistance in *Mycobacterium tuberculosis*. *Res Microbiol* 2004;155:830–4.
- Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoi SA, Allix C, Aristimunha L, Arora J, Baumanis V, Binder L, Cafrune P, Cataldi A, Cheong S, Diel R, Ellermeier C, Evans JT, Fauville-Dufaux M, Ferdinand S, Garcia de Viedma D, Garzelli C, Gazzola L, Gomes HM, Gutierrez MC, Hawkey PM, van Helden PD, Kadival GV, Kreiswirth BN, Kremer K, Kubin M, Kulkarni SP, Liens B, Lillebaek T, Ho ML, Martin C, Mokrousov I, Narvskaa O, Ngeow YF, Naumann L, Niemann S, Parwati I, Rahim Z, Rasolofa-Razanamparany V, Rasolonavalona T, Rossetti ML, Rüsch-Gerdes S, Sajduda A, Samper S, Shemaykin IG, Singh UB, Somoskovi A, Skuce RA, van Soolingen D, Streicher EM, Suffys PN, Tortoli E, Tracevska T, Vincent V, Victor TC, Warren RM, Yap SF, Zaman K, Portaels F, Rastogi N, Sola C. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiol* 2006;6:23.
- Marais BJ, Victor TC, Hesselink AC, Barnard M, Jordaan A, Brittle W, Reuter H, Beyers N, van Helden PD, Warren RM, Schaaf HS. Beijing and Haarlem genotypes are overrepresented among children with drug-resistant tuberculosis in the Western Cape province of South Africa. *J Clin Microbiol* 2006;44:3539–43.
- Shcherbakov D, Akbergenov R, Matt T, Sander P, Andersson DI, Böttger EC. Directed mutagenesis of *Mycobacterium smegmatis* 16S rRNA to reconstruct the *in vivo* evolution of aminoglycoside resistance in *Mycobacterium tuberculosis*. *Mol Microbiol* 2010;77:830–40.
- Spies FS, da Silva PE, Ribeiro MO, Rossetti ML, Zaha A. Identification of mutations related to streptomycin resistance in clinical isolates of *Mycobacterium tuberculosis* and possible involvement of efflux mechanism. *Antimicrobial Agents Chemother* 2009;52:2947–9.
- Sajduda A, Brzostek A, Poplawska M, Augustynowicz-Kopeć E, Zwolska Z, Niemann S, Dziadek J, Hillemann D. Molecular characterization of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Poland. *J Clin Microbiol* 2004;42:2425–31.
- Banerjee R, Allen J, Westenhouse J, Oh P, Elms W, Desmond E, Nitta A, Royce S, Flood J. Extensively drug-resistant tuberculosis in California, 1993–2006. *Clin Infect Dis* 2008;47:450–7.
- Streicher EM, Bergval I, Dheda K, Böttger EC, Gey van Pittius NC, Bosman M, Coetzee G, Anthony RM, van Helden PD, Victor TC, Warren RM. *Mycobacterium tuberculosis* population structure determines the outcome of genetics-based second-line drug resistance testing. *Antimicrobial Agents Chemother* 2012;56:2420–7.
- Davies AP, Billington OJ, Bannister BA, Weir WR, McHugh TD, Gillespie SH. Comparison of fitness of two isolates of *Mycobacterium tuberculosis*, one of which had developed multi-drug resistance during the course of treatment. *J Infect* 2000;41:184–7.
- Kurland CG, Hughes D, Ehrenberg M. In: *Escherichia coli and Salmonella: cellular and molecular biology*. Washington DC: American Society for Microbiology; 1996. p. 979–1004.
- Tubulekas I, Hughes D. Suppression of *rpsL* phenotypes by *tuf* mutations reveals a unique relationship between translation elongation and growth rate. *Mol Microbiol* 1993;7:275–84.
- Böttger EC, Springer B, Pletschette M, Sander P. Fitness of antibiotic-resistant microorganisms and compensatory mutations. *Nat Med* 1998;12:1343–4.
- van Soolingen D, de Haas PE, van Doorn HR, Kuijper E, Rinder H, Borgdorff MW. Mutations at amino acid position 315 of the *katG* gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium tuberculosis* in the Netherlands. *J Infect Dis* 2000;182:1788–90.
- Sander P, Springer B, Pramananan T, Sturmels A, Kappler M, Pletschette M, Böttger EC. Fitness cost of chromosomal drug resistance-conferring mutations. *Antimicrobial Agents Chemother* 2002;46:1204–11.
- Gillespie SH. Antibiotic resistance in the absence of selective pressure. *Int J Antimicrob Agents* 2001;17:171–6.