Impaired fitness of *Mycobacterium tuberculosis* resistant isolates in a cell culture model of murine macrophages

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**Objectives:** We analysed the ability of *Mycobacterium tuberculosis* clinical isolates to penetrate and grow inside murine macrophages as a surrogate of fitness.

**Methods:** Thirty-five drug-resistant and 10 drug-susceptible *M. tuberculosis* isolates were studied in a murine macrophage model from the J774.2 cell line in a 6 day protocol, performing semi-quantitative counts in Middlebrook 7H11 medium. The mycobacterial penetration index (MPI) after infection and the mycobacterial growth ratio (MGR) inside the macrophages were determined to evaluate the fitness of isolates.

**Results:** Isolates with the *katG* S315T mutation and multidrug-resistant (MDR) isolates had a significantly lower MGR compared with drug-susceptible isolates. The MPI of the isolates with the *katG* S315T mutation showed a significant decrease compared with the MPI of those without this mutation. A trend to significantly lower values was also observed on comparing the MPI of the MDR isolates with that of the drug-susceptible isolates and the isolates resistant to isoniazid.

**Conclusions:** The isoniazid-resistant and MDR isolates with mutations in the *katG* gene showed decreased multiplication inside murine macrophages, suggesting a lower fitness of *M. tuberculosis* with these resistance patterns.

**Keywords:** *M. tuberculosis*, *katG* mutation, *inhA* mutation, *rpoB* mutation, macrophage cultures

**Introduction**

In *Mycobacterium tuberculosis*, the agent of tuberculosis (TB), a loss of strain fitness due to changes produced by acquired drug resistance mutations has been described. Initial studies on the virulence of these strains in animal models revealed lesser growth of the resistant strains compared with that of susceptible strains. A turning point in fitness studies occurred when *katG* mutation at position 315 was associated with isoniazid resistance. This gene encodes catalase–peroxidase activity, which is key in preventing oxidative stress, and, thus, a mutation in this gene could affect the fitness of the mycobacteria. Isoniazid resistance mutations are observed mainly in the *katG* gene at position 315 and to a lesser extent in the intergenic zone *mabA-inhA*. In vivo and in vitro models, such as epidemiological studies, have analysed the behaviour of resistant and susceptible strains, determining whether the acquisition of mutations in these strains has led to a cost in fitness, albeit with no clear conclusions being drawn.

In the present study, we analysed the ability of *M. tuberculosis* clinical isolates to penetrate and grow inside murine macrophages as a surrogate of fitness.

**Materials and methods**

**Isolates, MICs and molecular resistance analysis**

Forty-five non-clustered *M. tuberculosis* clinical isolates (35 drug resistant to isoniazid and/or rifampicin, and 10 drug susceptible) from the...
Macrophage culture and M. tuberculosis inoculum preparation

J774.2 cell line murine macrophages (Sigma 85011428) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with L-glutamine, 4.5 g/L glucose (Lonza, Switzerland), 10% heat-inactivated fetal calf serum (Lonza), and a 100 U/mL penicillin and streptomycin mixture (Sigma), at 37°C in 5% CO₂ until exponential growth was achieved.

The isolates were grown in 7H9 MGIT medium (Becton Dickinson, MD, USA), supplemented with 0.25% Tween 80 (Merck, Germany) to avoid clump formation. When the MGIT was positive, the sample was centrifuged at 1174 g for 20 min, discarding the supernatant. Then, after adding 5 mm glass beads, the tube was shaken for 45 s and sonicated for 1 min. Lastly, clumps were disaggregated by 14 passages through a syringe needle (20G × 0.9 × 25 mm) and 4 passages through an insulin needle (27G × 0.4 × 12 mm). The inoculum was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard, making dilutions up to ~100000 cfu/mL. Fifty μL of this inoculum was plated on Middlebrook 7H11 medium and incubated at 37°C in 5% CO₂ for 21 days. Quantitative counts were made thereafter.

Infection protocol

The ability of infection of the different M. tuberculosis clinical isolates studied was determined using the mycobacterial penetration index (MPI) and the mycobacterial growth ratio (MGR). The MPI was defined as the percentage of mycobacterial cells penetrating inside the macrophages, and the MGR was defined as the intracellular growth index of the mycobacterial cells during the 6 day protocol.

Determination of MPI

Volumes of 1 mL of supplemented antibiotic-free DMEM containing 50000 murine macrophages were seeded in 24-well plates and incubated at 37°C in 5% CO₂. Each isolate studied was tested in duplicate. The macrophages were incubated for 3 h with the mycobacterial inoculum at a multiplicity of infection of 1:1. This point was considered day 0. The macrophages were washed three times with DMEM and lysis was performed by adding 0.5% NP40 detergent (Roche, Switzerland). After two washes, the cell lysate was seeded onto Middlebrook 7H11 plates and incubated at 37°C in 5% CO₂ for 3 weeks. Quantitative counts were made and the MPI was calculated as the proportion of cfu isolated in cell lysates on day 0 with respect to the cfu of the inoculum used.

Determination of MGR

A duplicate set of infected cells remained in incubation during the 6 day protocol. The medium was renewed after 1, 4 and 5 days of infection. The MGR was calculated as the ratio between the cfu of cell lysates on day 6 and the cfu of cell lysates on day 0.

Statistical analysis

The MPI and MGR of the different isolate groups were analysed using the Kruskal-Wallis test for more than two group comparisons. If the Kruskal-Wallis test showed significance, the Mann-Whitney U-test for two group comparisons was performed. Bonferroni adjustments for multiple comparisons among groups were used in this case. Statistical analyses were performed using SPSS 16, v02.

Results

Table 1 shows the molecular mechanisms of resistance and the MIC of isoniazid for the 35 drug-resistant isolates studied. Neither

Table 1. Characteristics of the 35 drug-resistant M. tuberculosis isolates

<table>
<thead>
<tr>
<th>Gene mutation</th>
<th>Type and location of mutations</th>
<th>Type of resistance</th>
<th>INH MIC (mg/L)</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>katG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S315T</td>
<td>INH</td>
<td>&lt;1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S315T</td>
<td>INH</td>
<td>1–9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>S315T</td>
<td>INH</td>
<td>≥10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>S315T</td>
<td>INH, STR</td>
<td>1–9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>S315T</td>
<td>INH, RIF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>≥10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>inhA</td>
<td>C–15T</td>
<td>INH</td>
<td>&lt;1</td>
<td>3</td>
</tr>
<tr>
<td>C–15T</td>
<td>INH</td>
<td>1–9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>rpoB</td>
<td>S531L</td>
<td>RIF</td>
<td>NA</td>
<td>5</td>
</tr>
<tr>
<td>S531L</td>
<td>INH, RIF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1–10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S531L</td>
<td>INH, RIF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≥10</td>
<td>1</td>
<td></td>
</tr>
<tr>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>S531L</td>
<td>RIF, STR</td>
<td>NA</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>katG and rpoB</td>
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<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>S315T and H526A</td>
<td>INH, RIF, STR, PZA</td>
<td>1–9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S315T and S531L</td>
<td>INH, RIF, STR, PZA</td>
<td>≥10</td>
<td>1</td>
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<td>S315T and S531L</td>
<td>INH, RIF</td>
<td>1–9</td>
<td>4</td>
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<tr>
<td>S315T and S531L</td>
<td>INH, RIF</td>
<td>&lt;1</td>
<td>1</td>
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<tr>
<td>S315T and S531L</td>
<td>INH, RIF, STR</td>
<td>≥10</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Wild-type

|                | INH, STR                       | ≥10                | 1              |                |
|                | INH                            | <1                 | 2              |                |
|                | INH                            | ≥10                | 1              |                |

ND, not done; NA, not applicable; INH, isoniazid; STR, streptomycin; RIF, rifampicin; PZA, pyrazinamide; EMB, ethambutol; S315T, serine→threonine change in the katG gene at position 315; C–15T, nucleotide change in the inhA gene; S531L, serine→leucine change in the rpoB gene at position 531; H526A, histidine→adenine change in the rpoB gene at position 526.

<sup>a</sup>Isolate resistant to rifampicin without a known rpoB mutation.
<sup>b</sup>Isolates resistant to isoniazid without a known katG or inhA mutation.
the MPI nor the MGR significantly differed according to the MIC ranges for the isolates studied.

**MPI according to the resistance pattern**

No significant difference was observed on comparing the MPI of the isolates, according to four categories: susceptible; isoniazid resistant; rifampicin resistant; and multidrug resistant (MDR). A trend to significantly lower values was observed on comparing the MPI of the MDR isolates with that of the drug-susceptible and the isoniazid-resistant isolates ($P = 0.042$ and $P = 0.036$, respectively).

**MPI according to the mutations associated with resistance**

A lower MPI was found in the resistant isolates with a mutation in the katG gene compared with those resistant without this mutation ($P = 0.009$), although no significant differences were observed on comparison with drug-susceptible isolates (Figure 1a).

**MGR according to the resistance pattern**

On comparing the above-mentioned categories, the MGR was significantly lower in the MDR isolates than in the drug-susceptible isolates ($P = 0.012$). The isoniazid-resistant isolates showed a trend to having a significantly lower MGR than drug-susceptible isolates ($P = 0.074$). Isolates resistant to only rifampicin did not significantly differ from drug-susceptible isolates.

**MGR according to the mutations associated with resistance**

A significantly lower MGR was found in the 21 isolates with a katG gene mutation compared with drug-susceptible isolates ($P = 0.013$). No significant difference was found when drug-susceptible isolates were compared with those without a katG gene mutation (Figure 1b). Furthermore, no significant difference was observed on comparing the 13 isolates with only a katG gene mutation and 8 isolates with mutations in the katG and rpoB genes with the drug-susceptible isolates ($P = 0.030$ and $P = 0.041$, respectively).

**Discussion**

The most important result of the present study is that isoniazid-resistant isolates with a katG gene mutation at position 315 and MDR isolates showed significantly lower growth in a murine macrophage model compared with drug-susceptible isolates.

The study of the molecular epidemiology of *M. tuberculosis* isolates is an excellent method to analyse TB transmission patterns by analysis of clusters and secondary cases and to indirectly study the fitness and virulence of clinical isolates. In this context, a significant number of isolates reported in different studies are worthy of comment. Burgos et al. detected fewer secondary cases from resistant isolates (MDR and only isoniazid resistant) than from susceptible isolates. In contrast, van Doorn et al. found that isolates with a katG gene mutation at position 315 were clustered as frequently as those susceptible to isoniazid. Likewise, Gagneux et al. indicated that isolates with the same mutation had as many secondary cases as those with a mutation in the inhA gene and suggested the same hypothesis as van Doorn et al., despite not comparing these isolates with drug-susceptible isolates.

Although in vitro models of macrophages from cell cultures provide an excellent opportunity to directly study the fitness of *M. tuberculosis* isolates, the literature is limited, making it difficult to draw clear conclusions. To our knowledge, the present study has analysed the capacity of penetration and multiplication of the largest number of susceptible and drug-resistant clinical isolates (10 and 35, respectively) according to their resistance pattern as well as their associated mutations reported to
date. Similar to the previously mentioned epidemiology studies, we observed that the fitness of MDR isolates decreased compared with that of drug-susceptible isolates.

We also observed an alteration in the fitness of the isoniazid-resistant isolates with mutation in the \( \text{katG} \) gene at position 315, in contrast to the previously reported results.\(^\text{7,8}\) This fitness alteration in isoniazid-resistant isolates with a mutation in the \( \text{katG} \) gene could be explained by some loss of catalase-peroxidase function.\(^\text{2}\) Discordance with epidemiological studies may be explained by the greater difficulty in detecting impaired fitness with indirect methods. In this sense, some aspects of molecular epidemiological analysis could contribute to increase the specificity of further studies on the epidemiological links among isolates from clusters, such as the use of secondary markers and contact tracing to determine whether patients belong to the same cluster or are not linked at all.

Recently, it has been reported that the dynamics of TB transmission may also be affected by the bacterial genetic background of \( M. \text{tuberculosis} \), which may influence the clinical localization, diagnostic aspects and the virulence of the isolates.\(^\text{10}\) Thus, future studies on the fitness of \( M. \text{tuberculosis} \) should consider these aspects.

In conclusion, isoniazid-resistant and MDR isolates with a \( \text{katG} \) gene mutation showed a decrease in their multiplication inside murine macrophages, suggesting a lower fitness of TB with these resistance patterns.

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Transparency declarations
None to declare.

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