Drug resistance increases tuberculosis (TB) mortality from 5 to 10% for drug-susceptible TB to 13 to 24% for multidrug-resistant tuberculosis (MDR-TB; defined as resistance to both isoniazid and rifampin) and 18 to 50% for extensively drug-resistant tuberculosis (XDR-TB; defined as MDR-TB plus resistance to fluoroquinolones and an injectable second-line drug) (1–3). The incidence of XDR-TB is rising worldwide and has now been detected in 69 countries (4). The worsening prognosis of XDR-TB compared to that of MDR-TB is strongly related to fluoroquinolone resistance (5), which is defined by resistance to at least 2 mg/liter of ofloxacin (6).

*Mycobacterium tuberculosis* resistance to fluoroquinolones depends on the substitution in the GyrA and/or GyrB subunits of the DNA gyrase, a catalytically active complex (GyrA<sub>2</sub>GyrB<sub>2</sub>) (7, 8). The level of resistance of DNA gyrase mutants depends both on the mutation and the fluoroquinolone tested (9). Usually, the level of resistance is higher for ofloxacin than for moxifloxacin (2 to 16 mg/liter and 1 to 8 mg/liter, respectively) (10–12). Consequently, some strains categorized as fluoroquinolone resistant still have moxifloxacin MICs lower than the usual 4 mg/liter peak serum level (13). We previously demonstrated, in the murine model, that the impact of DNA gyrase mutations on moxifloxacin monotherapy activity was related to the level of resistance generated by each mutation (14). However, as tuberculosis treatment relies on drug combinations, this may not be true in the case of multidrug therapy. Therefore, our next aim was to evaluate whether the level of fluoroquinolone resistance also impacts the efficacy of moxifloxacin-containing multidrug regimens.

**MATERIALS AND METHODS**

**Antimicrobials.** Drugs were obtained and solutions prepared as previously described (14, 15).

*Mycobacterium tuberculosis* strains. Four *M. tuberculosis* strains were used. Three isogenic mutant strains harboring DNA gyrase substitutions similar to those observed in clinical strains (12, 16), D94G and A90V in GyrA and D500N in GyrB, were obtained in a previous mouse study (15). The ofloxacin and moxifloxacin MICs were 0.5 and 0.25 mg/liter for the wild-type strain and were 4 and >8, >8 and 0.5, and 2 and 4 mg/liter for the strains harboring the GyrB D500N, GyrA A90V, and GyrA D94G substitutions, respectively (14). The wild-type reference H37Rv strain and the 3 mutant strains were grown from a mouse organ of a previous experiment as previously described (14, 15).

**Intravenous infection.** For each *M. tuberculosis* strain, 70 4-week-old female Swiss mice (Janvier Breeding Center, Le Genest Saint-Isle, France) were inoculated in the tail vein with 0.5 ml of a bacterial suspension prepared as previously described (17). The mean CFU counts were 6.8 for H37Rv, 6.4 log<sub>10</sub> for the GyrA D94G strain, 7.1 for the GyrA A90V strain, and 6.5 for the GyrB D500N strain. After infection, mice were randomized...
by day of sacrifice (Table 1). We followed the animal experiment guidelines of the Faculté de Médecine Pitié-Salpêtrière.

Chemotherapy. Treatment began 14 days after infection (day 0 [D0]). Three drugs were administered by gavage 5 days per week for 6 months at the following dosages: moxifloxacin, 100 mg/kg of body weight twice daily; ethionamide, 50 mg/kg; and pyrazinamide, 150 mg/kg once daily. Amikacin, 150 mg/kg, was administered by subcutaneous injection 5 days per week for the first 2 months. Drug dosages used were similar to those in our previous experiments (15, 18). The moxifloxacin dosage allowed for per week for the first 2 months. Drug dosages used were similar to those in our previous experiments (15, 18). The moxifloxacin dosage allowed for an area under the curve (AUC) value close to that found in humans taking our previous experiments (15, 18). The moxifloxacin dosage allowed for an area under the curve (AUC) value close to that found in humans taking our previous experiments (15, 18). The moxifloxacin dosage allowed for an area under the curve (AUC) value close to that found in humans taking our previous experiments (15, 18). The moxifloxacin dosage allowed for an area under the curve (AUC) value close to that found in humans taking our previous experiments (15, 18). The moxifloxacin dosage allowed for an area under the curve (AUC) value close to that found in humans taking our previous experiments (15, 18). The moxifloxacin dosage allowed for an area under the curve (AUC) value close to that found in humans taking our previous experiments (15, 18). The moxifloxacin dosage allowed for an area under the curve (AUC) value close to that found in humans taking our previous experiments (15, 18). The moxifloxacin dosage allowed for an area under the curve (AUC) value close to that found in humans taking our previous experiments (15, 18).

Assessment of treatment efficacy. We evaluated treatment efficacy by measuring lung CFU counts and the proportion of mice with culture-positive lung homogenates (i) during treatment and at the end of treatment, for assessing the bactericidal activity, and (ii) 3 months after treatment completion, for assessing sterilizing activity.

For each M. tuberculosis strain, 10 negative-control mice were untreated and kept under observation until death. Ten and 20 untreated mice were sacrificed, respectively, the day after infection (D – 13) and 14 days later, at the initiation of treatment (D0), to determine the initial CFU counts. Ten treated mice were killed after 2 months of treatment (M2), at the end of treatment (M6), and 3 months after treatment completion (M6 + 3) (Table 1).

Detection of second-step mutants after treatment completion. To check for in vivo selection of second-step mutants at M6 + 3, we used the direct proportion method. In addition to enumeration of the total number of CFU per organ, the undiluted organ suspensions were also plated onto Lowenstein-Jensen (LJ) medium containing oxolinic acid at 16 mg/liter for the GyrB D500N mutant strain and 64 mg/liter for the GyrA A90V mutant strain, i.e., concentrations 4 times their MICs before treatment (14). The D94G mutant was not screened for second-step mutations, as we did show in a previous experiment that they do not occur when moxi-floxacin is used alone, since the drug is inactive in this setting (14). Mutations leading to fluoroquinolone resistance were sought by sequencing the gyrA and gyrB genes of the bacilli isolated from the oxolinic-contain-

### Table 1: Scheme of experiment

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of mice untreated and kept under observation</th>
<th>No. of mice sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D – 13</td>
<td>D0</td>
</tr>
<tr>
<td>H37Rv</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>GyRS D500N strain</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>GyRS A90V strain</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>GyRS D94G strain</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

### RESULTS

Survival rates. All 40 untreated mice died between 10 and 116 days after infection. Mortality was similar among untreated mice infected with the M. tuberculosis H37Rv wild-type strain and those infected with any of the three derivative mutant strains (data not shown). Thus, the mutant and the wild-type strains of M. tuberculosis exhibited equal virulence in the mouse.

Bactericidal activity of the moxifloxacin-containing regimen. The mean log₁₀ CFU counts from lung homogenates and the proportions of mice with positive cultures are presented in Table 2.

The day after infection (D – 13), mean log₁₀ CFU counts in the lungs were 5.49 ± 0.18, 5.80 ± 0.18, 5.38 ± 1.0, and 5.46 ± 0.47 for mice infected with the H37Rv wild-type strain and the GyRS D500N, GyRS A90V, and GyRS D94G strains, respectively.

At D0, mean log₁₀ CFU counts in the lungs increased to 6.61 ± 0.42, 7 ± 0.23, 7.59 ± 0.78, and 6.51 ± 0.50 for mice infected with the H37Rv wild-type strain and the GyRS D500N, GyRS A90V, and GyRS D94G strains, respectively. Thus, CFU counts before treatment were different across all four groups (P = 3.6 × 10⁻⁶). Consequently, comparison between the four groups was based on the measurement of the decline in CFU counts across D0 and M2 within each group.

Between D0 and M2, mean log₁₀ CFU counts declined by 5.49, 4.52, 4.44, and 4.38 for mice infected with the H37Rv wild-type strain and the GyRS D500N, GyRS A90V, and GyRS D94G strains, respectively. These findings were mirrored in the proportion of mice with positive cultures. All mice infected with mutant strains (100%), but only three out of nine mice infected with the H37Rv wild-type strain (33%), had positive lung cultures, illustrating that initial bactericidal activity was significantly greater against the susceptible strain than the fluoroquinolone-resistant strains (P =

### Table 2: Mean lung CFU counts and proportion of mice with culture-positive lung homogenates at the initiation of treatment, during treatment, and 3 months after treatment completion among mice infected with the wild-type M. tuberculosis strain H37Rv and those infected with the GyRS D500N, GyRS A90V, and GyRS D94G mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Duration of treatment (mo)</th>
<th>0</th>
<th>2</th>
<th>6</th>
<th>Delay after treatment completion (+3 mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>0</td>
<td>6.61 ± 0.42 (20/20)</td>
<td>1.12  ± 0.16 (3/9)a</td>
<td>0 ± 0 (0/9)a</td>
<td>0 ± 0 (0/9)a</td>
</tr>
<tr>
<td>GyRS D500N strain</td>
<td>7 ± 0.23 (20/20)</td>
<td>2.48  ± 0.52 (10/10)</td>
<td>0.21 ± 0.58 (1/9)b</td>
<td>1.24 ± 1.73 (3/9)b</td>
<td></td>
</tr>
<tr>
<td>GyRS A90V strain</td>
<td>7.59 ± 0.78 (20/20)</td>
<td>3.15 ± 0.66 (10/10)</td>
<td>0.16 ± 0.17 (1/8)c</td>
<td>1.94 ± 2.12 (4/8)c</td>
<td></td>
</tr>
<tr>
<td>GyRS D94G strain</td>
<td>6.51 ± 0.50 (20/20)</td>
<td>2.13 ± 0.51 (7/7)d</td>
<td>0 ± 0 (2/8)d</td>
<td>2.42 ± 1.43 (6/7)d</td>
<td></td>
</tr>
</tbody>
</table>

a Fourteen mice died of gavage accidents before the date of sacrifice.

b Two mice died from tuberculosis before the date of sacrifice (before the initiation of treatment).

c CFU counts were not available for one sacrificed mouse because of technical problems.
LJ medium without ofloxacin, demonstrating the absence of a
toxicity in mice sacrificed at M6. No colony grew on medium containing ofloxacin at 16 and
D94G strain. CFU counts (M6) were close to 0 in all groups: 0 CFU for H37Rv, 45 CFU in 1
A90V, and GyrA D94G strains, respectively).

Limitation between drug resistance (14). The present study aimed to find out whether the
second-line antituberculous drugs.

Quinolone resistance would still be apparent when combined with
other differences between groups were not statistically significant
the GyrA A90V strain versus the GyrA D94G strain,
the group infected with the GyrB D500N, GyrA A90V, and GyrA D94G strains, re-
respectively, had culture-positive organs at this endpoint (Table 2).
The relapse rate was significantly lower for the group infected with the
H37Rv wild-type strain and three out of nine (33%),
four out of eight (50%), and six out of seven (86%) mice infected
with the GyrB D500N, GyrA A90V, and GyrA D94G strains, re-
respectively, had culture-positive organs at this endpoint (Table 2).
The difference in CFU counts between the latter group and the
wild-type-infected mice approached statistical significance (P = 0.27).
The difference between the group infected with the GyrB
D500N mutant strain and the group infected with the GyrA D94G
mutant strain closely approached significance (P = 0.06). The
other differences between groups were not statistically significant
(the GyrB D500N strain versus the GyrA A90V strain, P = 0.64;
the GyrA A90V strain versus the GyrA D94G strain, P = 0.28).

Detection of second-step mutants after treatment comple-
tion. No colony grew on medium containing ofloxacin at 16 and
64 mg/liter for mice sacrificed at M6 + 3. Furthermore, no mutation in gyrA or gyrB was found in individual colonies growing on LJ medium without ofloxacin, demonstrating the absence of a
second-step mutant selection in our experiment.

DISCUSSION
The consequence of detection of drug resistance has long been
considered an on/off scenario in tuberculosis: diagnosing resis-
tance was systematically leading to interruption of the drug. It is
now well known that, for all antituberculous drugs, the level of
resistance differs depending on the mutation generating resis-
tance. Low-level resistance has been described for isoniazid and
more recently for most antituberculous drugs (rifampin, etham-
butol, aminoglycosides, fluoroquinolones, and ethionamide) (9,
16, 22, 23). However, the in vivo consequences of low-level resis-
tance are still a matter of debate, as no clinical trial has assessed
rigorously this question, and very few preclinical data exist (24).

In a previous murine study, we demonstrated that moxifloxacin,
used alone, retains some activity in vivo against fluoroquin-
olone-resistant mutants and that this activity depends on the level
of resistance (14). The present study aimed to find out whether the
link between in vivo moxifloxacin activity and the level of fluoro-
quino lone resistance would still be apparent when combined with
second-line antituberculous drugs.

We first demonstrated that fluoroquinolone resistance dra-
matically reduces the sterilizing activity of a second-line regimen, even when composed of the most active available second-line antituberculous drugs (25–27) (Table 2). This result is consistent with those of previous in vivo studies showing that withholding moxifloxacin from a second-line regimen against the wild-type
H37Rv strain reduces both bactericidal and sterilizing activity (15,
18, 28) and with the numerous studies that have shown that fluoro-
quino lone resistance is a major prognostic factor for therapeu-
tic failure in cases of multidrug-resistant tuberculosis (5, 29, 30).

Second, our data suggested that the sterilizing activity of the
moxifloxacin-containing regimen decreases gradually against
strains displaying levels of resistance increasing from low to inter-
mEDIATE to high. These results are consistent with data obtained
for other bacterial species resistant to quinolones (31–33). Re-
garding the high-level resistant strain (the GyrA D94G strain), the
detrimental impact of fluoroquinolone resistance was expected, as
moxifloxacin alone is not active against this mutant in a murine
model (14). For the strain displaying an intermediate level of re-
sistance (the GyrA A90V strain), the relapse rate was significantly
higher than that observed with the H37Rv wild-type strain but
lower (although not statistically significant) than that seen with
the high-level resistant strain (Table 2). The sterilizing activity of
the moxifloxacin-containing regimen was not different for the
low-level resistant (the GyrB D500N strain) and the wild-type
the diagnosis of fluoroquinolone resistance being based mainly on
susceptibility testing of ofloxacin (2 mg/liter), such strains may
not be detected adequately. Therefore, our results suggest that,
among strains resistant to 2 mg/liter ofloxacin, precise identifica-
tion of the moxifloxacin level of resistance is required. This re-
quires either genotypic testing including gyrA and gyrB sequenc-
ing or moxifloxacin susceptibility testing on low and high drug
concentrations, which we suggest to be 2 mg/liter (9, 10, 38).

We demonstrated that although bactericidal activity against
resistant mutants compared to H37Rv was reduced after 2
months, this reduction did not appear to depend on the level of
fluoroquinolone resistance. This reinforces that in murine studies,
bactericidal activity of drug combinations is not predictive of ster-
ilizing activity (39, 40). However, here, the bactericidal activity
was assessed only after 2 and 6 months. An intermediate time
point may have been able to show a difference between the mutant
strains. Also, when analyzing this and the apparently contradic-
tory results regarding the sterilizing activity, one must bear in
mind that, in our study, moxifloxacin was combined with the
most active second-line drugs: amikacin, ethionamide, and pyr-
azonamide (25–27). Therefore, the fact that there was no differ-
ce in bactericidal activity against the three mutant strains after 2
and 6 months of treatment was probably due to the high activity
of these three drugs. Since the second-line drugs still available to
treat XDR-TB, such as cycloserine, para-aminosalicylic acid
(PAS), or linezolid, are less active in clinical practice than those
used in our study, the impact of fluoroquinolone resistance would
probably be more important if moxifloxacin were combined with
these drugs.

A caveat must be underlined when interpreting the results of
this experiment. As the peak serum level of 100 mg/kg of moxi-
floxacin in mice is higher than that of 400 mg in humans, it could
be possible that the activity against fluoroquinolone-resistant mu-

P = 0.0031, P = 0.0031, and P = 0.011 for the GyrB D500N, GyrA
A90V, and GyrA D94G strains, respectively).

Finally, the CFU counts in the lungs at the end of treatment
(M6) were close to 0 in all groups: 0 CFU for H37Rv, 45 CFU in 1
mouse (11%) for the GyrB D500N strain, 3 CFU in 1 mouse (13%)
for the GyrA A90V strain, and 1 CFU in 2 mice (25%) for the GyrA
D94G strain. CFU counts (P = 0.56) and the proportion of mice
with positive cultures (P = 0.2 to 1) were not significantly differ-
et among those infected with the respective four strains. Thus,
bactericidal activity was not different against the susceptible strain
and the fluoroquinolone-resistant strains after 6 months of the
moxifloxacin-containing second-line regimen, despite an assess-
ment at an earlier time point that had identified differences.

Relapse after treatment completion. Three months after
treatment completion (M6 + 3), the relapse rates were related to
the drug resistance level. Indeed, no mice infected with the M.
tuberculosis H37Rv wild-type strain and three out of nine (33%),
four out of eight (50%), and six out of seven (86%) mice infected
with the GyrB D500N, GyrA A90V, and GyrA D94G strains, re-
spectively, had culture-positive organs at this endpoint (Table 2).
The relapse rate was significantly lower for the group infected with the
H37Rv wild-type strain than for the groups infected with the
GyrA A90V and GyrA D94G mutant strains (P = 0.03 and P = 0.0009,
respectively), whereas it was not different from the group
infected with the GyrB D500N mutant strain (P = 0.2). However,
the difference in CFU counts between the latter group and the
wild-type-infected mice approached statistical significance (P = 0.077).
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We first demonstrated that fluoroquinolone resistance dra-

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tants is exaggerated in mice compared to that in humans. How-
ever, we do not believe this is the case, since we demonstrated in a previous experiment that the main pharmacokinetic driver of moxifloxacin activity against fluoroquinolone-resistant mutants was the $C_{\text{max}}$ (14).

Does this study support the WHO recommendation to include a later-generation fluoroquinolone, e.g., moxifloxacin, in a second-line TB regimen even in the case of fluoroquinolone-resistant tuberculosis (25, 41)? On one hand, there seems to be an impact of the level of resistance on the relapse rate, which supports the use of later-generation fluoroquinolone, at least against strains with low-level resistance (moxifloxacin MIC of less than 2 mg/liter). On the other hand, compared to the wild-type strain, the low-level resistance strain had a bacillary load higher at 2 months and almost significantly higher 3 months after the end of treatment, which means that even if a later-generation fluoroquinolone can improve treatment, it will not reverse fluoroquinolone resistance. Clearly, further studies need to be done, particularly to compare multidrug regimens with or without moxifloxacin to quantify more precisely the contribution of moxifloxacin. It would also be useful to compare multidrug regimens, including moxifloxacin at several dosages, to regimens without moxifloxacin in order to determine more precisely the impact of increasing moxifloxacin dosages on treatment efficacy. Indeed, since moxifloxacin activity depends on the AUC/MIC ratio (14, 42), we expect that doubling the dosage in humans (800 mg) could substantially improve the activity of a moxifloxacin-containing second-line drug regimen against strains with intermediate resistance. Such a dosage has already been used and seems safe (43).

Interestingly, our experiment could serve as a model for further design of studies aiming at determining the impact of different levels of resistance to other antituberculous drugs, which would help in implementing optimal treatments of patients with XDR-TB when the bacillus is resistant to all, or almost all, antituberculous drugs.

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