In Vitro Susceptibilities of *Rickettsia rickettsii* and *Rickettsia conorii* to Roxithromycin and Pristinamycin

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In vitro susceptibilities of *Rickettsia rickettsii* and *Rickettsia conorii* to roxithromycin, pristinamycin, and the pristinamycin compounds, P1 and P2, were determined by a dye uptake assay and a plaque assay. The MICs were 1 μg/ml for roxithromycin, 2 μg/ml for pristinamycin, >256 μg/ml for P1, and 2 μg/ml for P2. Compounds P1 and P2 did not share synergetic activity. The toxicity of each compound was determined by a dye uptake assay. Toxic concentrations were 128 μg/ml for roxithromycin, 32 μg/ml for pristinamycin, >256 μg/ml for P1, and 32 μg/ml for P2. Roxithromycin and pristinamycin could be useful in the treatment of Rocky Mountain spotted fever and Mediterranean spotted fever.

*Rickettsia rickettsii* and *Rickettsia conorii* are obligate intracellular bacteria. Their morphological characteristics and biochemical compositions are similar to those of gram-negative bacteria. Antibiotics active against these rickettsial species must penetrate intracellularly. Both rickettsial species are susceptible in vitro to the following antibiotics: tetracycline, chloramphenicol, and rifampin (10); ofloxacin (9); ofloxacin (D. Raoult, M. Yeaman, and O. Baca, Rev. Infect. Dis., in press); and ciprofloxacin (7).

*R. rickettsii* is the etiological agent of Rocky Mountain spotted fever (RMSF), and *R. conorii* is the etiological agent of Mediterranean spotted fever (MSF). The recommended treatment for both diseases is tetracycline or chloramphenicol (2, 3). These two antibiotics are toxic during pregnancy and childhood and should not be used in these situations. In such cases, macrolide antibiotics are proposed as alternative therapy, but the susceptibilities of rickettsiae to these compounds are highly variable.

We present the results of in vitro tests of the susceptibilities of *R. rickettsii* and *R. conorii* to the macrolide antibiotic roxithromycin and to the streptogramin antibiotic pristinamycin and its compounds, P1 and P2 (4).

**Bacterial strains.** *R. rickettsii* (Sheila Smith strain) and *R. conorii* (Moroccan strain) were cultivated on Vero cells. The titer of the inoculum was determined by plaque assay (12) and adjusted to 4 × 10⁴ PFU/ml in Eagle minimum essential medium (MEM).

**Antibiotics.** A stock solution of each antibiotic was prepared at a concentration of 1 μg/ml. Roxithromycin (Laboratoires Roussel, Paris, France) was diluted in phosphate-buffered saline, and pristinamycin and its compounds, P1 and P2 (Laboratoires Specia, Paris, France), were diluted at 60% methanol. The solutions were passed through 0.22-μm-pore-size filters and were stored at −20°C. The working dilutions were made daily in MEM.

**Susceptibility tests.** (i) **Dye uptake assay** (10). A suspension of 1.5 × 10⁸ Vero cells per ml was prepared in a solution of Eagle MEM, 5% fetal calf serum, and 2 mM glutamine. One hundred microliters of the suspension was dispensed in each well of a 96-well, flat-bottomed microdilution culture plate (CEB, Nancy, France). The inoculum was added in a final volume of 50 μl per well: 2,000 PFU of rickettsiae was added in each well of the first line, 200 PFU per well was added in the second line, and 20 PFU per well was added in the third line. The fourth line was used as a cell control, and no rickettsiae were added. Two thousand PFU of rickettsiae was added in each well of the eight remaining lines, which were then used for antibiotic assay. Antibiotic was dispensed in a volume of 50 μl per well. Eight serial dilutions from 64 to 0.5 μg/ml were tested. Additional dilutions were tested if necessary. The plate was then incubated for 4 days at 36°C in a 5% CO₂ incubator. The medium was then discarded, and 50 μl of neutral red dye (0.15% in saline, pH 5.5) was placed in each well. The plate was incubated for 1 h at 36°C. The wells were washed three times with phosphate-buffered saline, and the incorporated red dye was extracted by using 100 μl of phosphate ethanol buffer (10% ethanol in phosphate-buffered saline, pH 4.2) per well.

The optical density at 492 nm (OD₄₉₂) of the solution was determined with a spectrophotometer (Flow Laboratories, McLean, Va.). A concentration of antibiotic was estimated as efficient if the mean OD₄₉₂ of the line was between those of the cell control line and the line with 20 PFU per well.

(ii) **Plaque assay.** Five milliliters of a suspension of 1.5 × 10⁶ Vero cells per ml was dispensed in each tissue culture petri dish (diameter, 60 mm; Corning Glass Works, Corning, N.Y.). Dishes were incubated for 24 h at 36°C in a 5% CO₂ incubator. The medium was then discarded, and the confluent cell monolayers were inoculated with 1 ml of a solution containing 4 × 10⁴ PFU of rickettsiae. After incubation for 1 h at 22°C, infected cells were overlaid with 5 ml of a medium containing MEM, 2% fetal calf serum, and 0.5% agar. Antibiotics were added to a final concentration of 0 (positive control), 0.5, 1, 2, and 4 μg/ml. Additional dilutions were tested if necessary. The dishes were incubated for 4 days at 36°C in a 5% CO₂ incubator and were then stained by adding a second overlay containing MEM, 0.5% agar, and 0.01% neutral red dye. Three experiments were carried out at each antibiotic concentration.

**Toxicity tests.** The toxicity of each antibiotic for Vero cells was determined by using a dye uptake assay similar to that described above. A suspension of 1.5 × 10⁶ Vero cells per ml in a solution of MEM, 5% fetal calf serum, and 2 mM glutamine was prepared. One hundred microliters of the suspension was dispensed in each well of a 96-well, flat-bottomed microdilution culture plate. The antibiotic was...
added in a final volume of 100 µl per well. Eleven serial dilutions from 512 to 0.5 µg/ml were tested per plate; the first line was used as a cell control. The plate was incubated for 4 days at 36°C in a 5% CO₂ incubator. The medium was then removed, and 50 µl of neutral red dye was dispensed into each well.

The plate was incubated for 60 min at 36°C and washed three times with phosphate-buffered saline. Incorporated red dye was extracted by using 100 µl of phosphate ethanol buffer per well. The OD₄₉₀ of the solution was determined by using a multichannel spectrophotometer. A concentration of antibiotic in a given line was considered toxic when the mean OD₄₉₀ of the line was lower than the mean OD₄₉₀ of the cell control line. Each antibiotic was tested on three plates.

**Susceptibility tests.** The results of the susceptibility tests are presented in Table 1. Toxicity for Vero cells was noted at concentrations of 128 µg/ml for roxithromycin, 32 µg/ml for pristinamycin, >256 µg/ml for P1, and 32 µg/ml for P2. Results of susceptibility tests were the same in the dye uptake assay and the plaque assay, as previously reported (10). The MICs of each antibiotic were the same for *R. rickettsii* and *R. conorii*. The MICs were 1 µg/ml for roxithromycin, 2 µg/ml for pristinamycin, >256 µg/ml for P1, and 2 µg/ml for P2. The toxic concentration of each drug except P1 was at least 16 times higher than the MIC; thus, toxicity for Vero cells did not interfere with the susceptibility tests. Pristinamycin results from the combination of two compounds, P1 and P2. P1 was ineffective against both rickettsiae species, while the MIC of P2 was the same as that of pristinamycin, i.e., 2 µg/ml. There was no synergy of P1 and P2 against either species tested.

**Discussion and conclusions.** Chloramphenicol and tetracyclines are the recommended treatments for both RMSF and MSF. For both *R. rickettsii* and *R. conorii*, the MIC of chloramphenicol is 0.5 µg/ml, the MIC of doxycycline is 0.06 µg/ml, and the MIC of tetracycline is 0.25 µg/ml (10). New quinolone compounds are also effective. The MICs of pefloxacin are 0.5 µg/ml for *R. conorii* and 1 µg/ml for *R. rickettsii* (9). The MICs of ciprofloxacin are 0.25 µg/ml for *R. conorii* and 1 µg/ml for *R. rickettsii* (7). Ciprofloxacin (6), pefloxacin, and ofloxacin (1) have been successfully used in treating MSF. However, the toxicities of these effective antibiotics limit their use during pregnancy and childhood. Chloramphenicol causes aplastic anemia and gray syndrome in young children; tetracyclines given during pregnancy have teratogenic effects, and they stain the teeth yellow when given during childhood. The safety of new quinolone compounds for pregnant women is not ensured. Macrolide antibiotics have been proposed as alternative therapy in these situations. Erythromycin is effective in suppressing lethality for chicken embryos (11), but its MICs are 4 µg/ml for *R. conorii* and 8 µg/ml for *R. rickettsii*, as determined by plaque assay (10), and thus it is ineffective in humans. The superiority of tetracycline versus erythromycin in the treatment of MSF during childhood has been clearly established (5). Spiramycin has MICs of 16 µg/ml for *R. conorii* and 32 µg/ml for *R. rickettsii* (8) and should not be used in the treatment of MSF or RMSF. Josamycin has a MIC of 1 µg/ml for both rickettsial species. It has been successfully used in two cases of MSF during pregnancy (D. Raoult, unpublished data), and it has been reported to be as effective as doxycycline and superior to erythromycin in the treatment of MSF (F. Bella, B. Font, T. Munoz, J. A. Serrano, E. Espejo, S. Uriz, E. Gaban, and F. Segura, Program Abstr. 4th Eur. Congr. Clin. Microbiol., abstr. no. 604, 1989). This confirms the in vitro data. These facts support the idea that in vitro results of susceptibility in this model are correlated with in vivo results.

The present work leads us to think that roxithromycin is effective, with a MIC of 1 µg/ml for both rickettsial species. These results confirm the heterogeneity of susceptibility of spotted-fever group rickettsiae to macrolide antibiotics; the reasons for this heterogeneity are not known. Each compound of this group must be tested in vitro to determine the susceptibilities of rickettsial strains. As for the streptogramin molecule, pristinamycin is effective in vitro against *R. rickettsii* and *R. conorii*. Prudent clinical trials must confirm the efficiency of roxithromycin and pristinamycin in the treatment of RMSF and MSF before they can be recommended for use during pregnancy and childhood.

**TABLE 1. Susceptibility of *R. rickettsii* and *R. conorii* to antibiotics**

<table>
<thead>
<tr>
<th>Drug (reference)</th>
<th>MIC (µg/ml) for strain with assay type</th>
<th><em>R. rickettsii</em> Sheila Smith</th>
<th><em>R. rickettsii</em> Moroccan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dye uptake</td>
<td>Plaque</td>
</tr>
<tr>
<td>Chloramphenicol</td>
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<td>0.5</td>
<td>0.5</td>
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<td>Doxycycline</td>
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<td>0.06</td>
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<td>Tetracycline</td>
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<td>0.25</td>
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<tr>
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<td>0.5</td>
<td>1</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
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<td>1</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td></td>
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<td>1</td>
</tr>
<tr>
<td>Pristinamycin</td>
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<td>2</td>
</tr>
<tr>
<td>P1 (PW)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>P2 (PW)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
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* PW, Present work.

**LITERATURE CITED**

9. Raoult, D., P. Roussellier, G. Vestris, V. Galicher, R. Perez, and

