Bactericidal Activity of Antibiotics Against Legionella micdadei (Pittsburgh Pneumonia Agent)

JOHN N. DOWLING,1,2* ROBBIN S. WEYANT,3 AND A. WILLIAM PASCULLE2,3

Departments of Medicine1 and Pathology,2 School of Medicine, and Department of Microbiology,3 Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Received 16 March 1982/Accepted 1 June 1982

Pneumonia due to Legionella micdadei (Pittsburgh pneumonia agent) was first described in 1979 (9, 12, 15). The etiological agent was isolated by Pasculle et al. (12) and proved to be identical to the bacterium cultivated in eggs by Tatlock in 1943 (7). Since the clinical features of pneumonia due to L. micdadei and L. pneumophila are quite similar (3), the two diseases can be differentiated only by identification of the causative bacterium or by serological techniques. Fortunately, erythromycin and rifampin appear to be efficacious for the therapy of both Pittsburgh pneumonia and Legionnaires disease (3, 19). There is also evidence that erythromycin and rifampin are efficacious against these legionellae in ovo and in a guinea pig infection model (5, 8, 9).

All isolates of L. pneumophila (4, 6, 16–18) and L. micdadei (6, 10) examined thus far have been inhibited in vitro by readily achievable concentrations of chloramphenicol and the aminoglycosides, as well as erythromycin and rifampin. L. micdadei, which does not produce a β-lactamase, is also exquisitely susceptible to penicillins and cephalosporins (10). However, antibiotics other than erythromycin and rifampin which inhibit L. micdadei and L. pneumophila in vitro have not consistently proven to be effective in vivo (5, 8, 9). One possible explanation for these discrepancies between in vivo and in vitro results is that some antimicrobial agents which inhibit the legionellae do not kill these bacteria. To examine this possibility, we determined the bactericidal activity of five antibiotics for L. micdadei by time-kill curves.

(This work was presented in part at the 82nd Annual Meeting of the American Society for Microbiology, Atlanta, Ga., 7 to 12 March 1982.)

MATERIALS AND METHODS

Media. Buffered charcoal-yeast extract (BCYE) agar was made as described previously (11). Buffered yeast extract broth (BYEB) was prepared as described (14), except that 10 g liter of acetamidoethanesulfonic acid (Research Organics, Cleveland, Ohio) was added.

Bacteria. A single strain, EK, of L. micdadei (ATCC 33204; 11) was employed. Stock bacteria for time-kill experiments consisted of L. micdadei passed two or three times through BYEB and frozen at −70°C. Staphylococcus aureus (ATCC 25923) was utilized as a control strain.

Antibiotics. All antibiotics were obtained as laboratory standard powders or solutions. Stock solutions of each antibiotic were made with appropriate solvents and diluents (1).

Killing curve studies. For each experiment, a 0.3-ml sample of the stock suspension of L. micdadei was thawed at 25°C and added to 30 ml of BYEB. After incubation for about 20 h on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 200 rpm in a 35°C water bath, the bacterial cell concentration was adjusted to about 109 bacteria per ml by dilution of the broth culture with BYEB until it spectrophotometrically matched a MacFarland no. 3 standard. This standardized inoculum was diluted 1:10 with BYEB, and 1.0 ml of this dilution was added to 9.0 ml of BYEB in 25-ml flasks to produce an initial concentration of about 107 bacteria per ml. Antibiotics were then added in concentrations ranging from 0.008 to 4.0 μg/ml in twofold increments, and the flasks were incubated at 35°C on the rotary shaker. Growth control flasks containing no antibiotic were included in each experiment. Before the addition of the antibiotic and at intervals up to 72 h thereafter, 0.5-ml samples were removed from each flask, including the growth
control. Appropriate 10-fold dilutions of each sample were made in sterile distilled water, and 0.1 ml of each dilution was plated in duplicate on BCYE agar plates by the spread-plate technique. After 72 h of incubation, the colonies were counted, and the geometric mean number of colony-forming units (CFU) was calculated for each time point. Duplicate sets of flasks with and without antibiotic were similarly inoculated with S. aureus, and the S. aureus minimal inhibitory concentration (MIC) was read visually at 24 h to serve as a control for antibiotic activity.

RESULTS

Preliminary experiments showed that reliable growth and killing curves were obtained only if the inoculum consisted of BYEB-passed bacteria which were in the exponential phase of growth. In addition, experiments in which the inoculum titered less than $10^6$ to $10^7$ bacteria per ml often produced growth curves with extended lag phases before growth resumed and, thus, uninterpretable killing curves. Such experiments were discarded. At least two experiments in which the antibiotic-free control showed loga-

![Diagram 1](http://aac.asm.org/)

**FIG. 1.** Killing curves for *L. micdadei* with erythromycin. Symbols: $\bullet$, 0 $\mu$g/ml; $\square$, 0.031 $\mu$g/ml; $\times$, 0.063 $\mu$g/ml; $\triangle$, 0.125 $\mu$g/ml; $\blacksquare$, 0.25 $\mu$g/ml; $\bigcirc$, 0.5 $\mu$g/ml; $+$, 1.0 $\mu$g/ml; $\nabla$, 2.0 $\mu$g/ml; $\blacklozenge$, 4.0 $\mu$g/ml.

rithmic growth with the peak titer attained at 24 or 30 h were done with each antibiotic; the results of a representative experiment with each antibiotic are shown in Fig. 1 to 5.

Killing curves with erythromycin are shown in Fig. 1. Increasing concentrations from 0.5 to 4.0 $\mu$g/ml, the highest tested, produced a gradually increasing rate of bacterial killing by erythromycin. However, at 0.5 $\mu$g of erythromycin per ml, killing was observed for 48 h, but exponential growth at a rate essentially equal to that of the antibiotic-free control ensued thereafter. This same pattern was also observed at a concentration of 0.5 $\mu$g/ml in a second experiment. By plating the cells from this concentration onto BCYE agar containing various concentrations of erythromycin up to 50 $\mu$g/ml, it was shown that the bacteria which regrew at 72 h were as susceptible to erythromycin as the original inoculum.

Even the lowest concentration of rifampin tested, 0.008 $\mu$g/ml, totally inhibited the growth of *L. micdadei* over the first 30 h (Fig. 2). Rifampin at a concentration of 0.125 $\mu$g/ml or above was rapidly bactericidal. Late regrowth of bacteria was seen at 0.008 $\mu$g of rifampin per ml in this experiment and at 0.001 $\mu$g/ml in two
antibiotic are shown in Table 1 and compared with the MICs of *L. micdadei* EK, determined previously by us, using a plate-dilution assay on BCYE agar (10).

The MICs of the *S. aureus* control strain obtained by broth dilution in BYEB are compared with those published for Mueller-Hinton broth (13) in Table 2. The MICs for rifampin, penicillin G, and cephalothin showed little difference between the two media. The MIC of *S. aureus* appeared to be 5-fold higher for erythromycin, and 10-fold higher for gentamicin, in the yeast extract base medium when compared with Mueller-Hinton broth.

**DISCUSSION**

These studies showed BYEB to be an acceptable medium for determining the bactericidal activity of erythromycin, rifampin, penicillin G, cephalothin, and gentamicin for *L. micdadei*. However, there were discrepancies between the apparent MICs of erythromycin and gentamicin for the control *S. aureus* in BYEB and those obtained in Mueller-Hinton broth. This may have been related to the higher inoculum con-

---

**FIG. 3.** Killing curves for *L. micdadei* with penicillin G. Symbols: ○, 0 μg/ml; □, 0.016 μg/ml; ×, 0.031 μg/ml; Δ, 0.063 μg/ml; □□, 0.125 μg/ml; ○, 0.250 μg/ml.

Other separate experiments. The bacteria which showed regrowth were resistant to 100 μg of rifampin per ml. Thus, in these three experiments, rifampin-resistant variants were selected out by low concentrations of rifampin.

The killing curves for penicillin G (Fig. 3) and cephalothin (Fig. 4) were very similar. Some inhibition was seen at low levels, and increasing rates of killing were noted above 0.063 μg of penicillin per ml and 0.5 μg of cephalothin per ml.

*L. micdadei* was rapidly killed by relatively low concentrations of gentamicin (Fig. 5). Figure 5 also demonstrates that the killing rate approached a maximum at higher concentrations of each antibiotic. The rate of killing did not increase beyond this maximum regardless of the concentration of a particular antibiotic employed.

For the purpose of comparison of the data from the time-kill curves with the inhibition of *L. micdadei* by the same antibiotics, the minimal bactericidal concentration (MBC) in each experiment was defined as the lowest antibiotic concentration which produced 99.9% or greater killing within 24 h. The geometric mean MBCs calculated from all experiments done with each
centrations or the lower pH of the medium employed in our studies. Thus, although the measured MBCs of erythromycin and gentamicin for *L. micdadei* were quite low, the actual MBCs may be even lower.

Erythromycin and rifampin were found to be bactericidal, as well as bacteriostatic, for *L. micdadei* in vitro at low concentrations. Erythromycin and rifampin were also highly effective in ovo (9) and have proven efficacious in the treatment of patients with Pittsburgh pneumonia (3, 19). On the basis of all in vitro studies and in vivo observations, erythromycin and rifampin remain the agents of choice for the treatment of Pittsburgh pneumonia.

The results of previous agar dilution susceptibility studies (10) showed that *L. micdadei* was inhibited by low concentrations of penicillin G, cephaplatin, and gentamicin, as well as erythromycin and rifampin. The present study established that these three antibiotics also kill *L. micdadei* in vitro at readily achievable concentrations. Furthermore, the MBC/MIC ratios for these antibiotics were as favorable as the ratios for erythromycin and rifampin (Table 1). However, a number of treatment failures have been reported among patients with *L. micdadei* pneumonia who were treated with β-lactam or aminoglycoside antibiotics or both (9, 15). In addition, these antibiotics failed to protect embryonated eggs against a lethal challenge of *L. micdadei* (9). We conclude that the ineffectiveness of β-lactam and aminoglycoside antibiotics against *L. micdadei* in vivo cannot be explained on the basis of a lack of bactericidal action.

Presumably, those antibiotics which are bactericidal in vitro but ineffective in vivo must not be active at the intracellular location of the legionellae. However, Bacheson and her coworkers (2) reported that gentamicin was more effective than erythromycin in killing *L. pneumophila* within human embryonic lung fibroblast cells. If these results are confirmed, it raises the question of whether the precise cell type(s) in which the legionellae reside must be taken into account in future studies concerning the intracellular killing of these bacteria.

### ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-17047 from the National Institute of Allergy and Infectious Diseases.

We thank David A. McDevitt for excellent technical assistance and Carl W. Norden for his gift of rifampin.

### LITERATURE CITED


### TABLE 1. Comparison of MICs and MBCs of antibiotics for *L. micdadei* EK

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (μg/ml)</th>
<th>MBC (μg/ml)</th>
<th>MBC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>0.59</td>
<td>4.59</td>
<td>7.8</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.05</td>
<td>0.13</td>
<td>2.5</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>≤0.06</td>
<td>0.25</td>
<td>≥4.2</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>0.46</td>
<td>2.52</td>
<td>5.5</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.25</td>
<td>0.25</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*a* MIC as determined by agar dilution (10).

*b* MBC defined as ≥99.9% killing within 24 h.

### FIG. 5. Killing curves for *L. micdadei* with gentamicin.

Symbols: ○, 0 μg/ml; □, 0.031 μg/ml; Δ, 0.063 μg/ml; ○, 0.125 μg/ml; (---), 0.25 μg/ml; (---), 0.5 μg/ml; (---), 1.0 μg/ml; (---), 2.0 μg/ml.

### TABLE 2. MICs of antibiotics for *S. aureus* (ATCC 25923) in BYEB broth and Mueller-Hinton broth

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>BYEB</th>
<th>Mueller-Hinton broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>2.00</td>
<td>0.4</td>
</tr>
<tr>
<td>Rifampin</td>
<td>≤0.06</td>
<td>0.1</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.04</td>
<td>0.1</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>0.19</td>
<td>0.1</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1.33</td>
<td>0.1</td>
</tr>
</tbody>
</table>

This work was supported by Public Health Service grant AI-17047 from the National Institute of Allergy and Infectious Diseases.

We thank David A. McDevitt for excellent technical assistance and Carl W. Norden for his gift of rifampin.

### LITERATURE CITED


