Isolation and Preliminary Characterization of Erythromycin-Resistant Variants of *Legionella micdadei* and *Legionella pneumophila*

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Erythromycin-resistant *Legionella* spp. variants were obtained by a single passage of the naturally occurring bacteria on medium containing various concentrations of erythromycin. By disk diffusion susceptibility testing, at least three different phenotypic patterns of cross-resistance to macrolide, lincosamide, and streptogramin B antibiotics were observed among the 26 erythromycin-resistant strains examined.

All of the legionellae examined have been found to be inhibited in vitro by readily achievable concentrations of erythromycin (3–5, 11, 13, 14), but it is not known whether antimicrobial resistance will arise within this genus in the future. There is no prior experience with the development of erythromycin resistance among natural isolates of gram-negative aerobic bacilli in clinical circumstances, since these bacteria are usually inherently resistant because erythromycin fails to penetrate the cell envelope (7). We describe herein the isolation of *Legionella* spp. variants with various degrees of erythromycin resistance and the patterns of cross-resistance displayed to other agents in the macrolide, lincosamide, and streptogramin B (MLS) classes of antibiotics.

Bacterial suspensions of *Legionella micdadei* (ATCC 33204) and *Legionella pneumophila*, serogroup 1, of approximately 10^7 CFU were plated on buffered charcoal-yeast extract (BCYE) agar (12) containing 2.5 or 5.0 μg of erythromycin lactobionate (Abbott Pharmaceuticals, North Chicago, Ill.) per ml, and about 10^9 CFU was plated on medium containing 10, 50, or 100 μg of erythromycin per ml. After 1 to 2 weeks of incubation, individual colonies were passed to erythromycin-free BCYE agar. The variants isolated were found to be stably resistant after multiple passages on erythromycin-free medium.

The MICs for erythromycin of the resistant variants, as determined by plate dilution on BCYE agar (11), and the susceptibility to MLS antibiotics by disk diffusion testing (1) are shown in Table 1. All of the resistant variants demonstrated cross-resistance to oleandomycin and, with one exception, to vernamycin B. One *L. micdadei* variant isolated from medium with 5.0 μg of erythromycin per ml was as susceptible to vernamycin B as the parent strain. When numerous plates of BCYE agar containing 10, 50, or 100 μg of erythromycin per ml were each inoculated with 10^9 *L. micdadei* cells, only a single colony was recovered from one containing 50 μg of erythromycin per ml. The calculated frequency of *L. micdadei* variants resistant to ≥ 10 μg of erythromycin per ml was 1.0×10^{-10} . This *L. micdadei* strain was found to have an erythromycin MIC of >100 $\mu\text{g}/\text{ml}$ and demonstrated no zones of inhibition around erythromycin, oleandomycin, rosaramicin, and tylosin disks. The disk susceptibility of the high-level resistant strain with

vernamycin B was determined on five occasions employing a separate stock sample that had been frozen at -70°C . The mean vernamycin zone diameter for the first two determinations was modestly smaller than that of the parent strain, but this difference did not reach statistical significance. When tested three additional times, there was no zone of inhibition around the vernamycin B disk, a highly significant difference from the parent strain run in parallel. These results can be explained by the assumption that the stock culture of the high-level resistant variant contained bacteria with two different genotypes. That a single intermediate-level resistant variant did not develop cross-resistance to vernamycin B confirmed that *L. micdadei* strains with at least two slightly different mutations were selected on exposure to erythromycin. Whether a change in phenotypic expression of one of the genotypes took place during storage of the stock culture at -70°C or an additional mutational event occurred during that time could not be distinguished.

No *L. pneumophila* variants grew on agar containing 5 μg of erythromycin per ml. From numerous plates containing 10, 50, or 100 $\mu\text{g}/\text{ml}$, a single *L. pneumophila* variant was recovered from 10 $\mu\text{g}/\text{ml}$, yielding a calculated frequency of 7.1×10^{-11} for *L. pneumophila* variants resistant to ≥ 10 μg of erythromycin per ml. The MIC for erythromycin of this high-level resistant variant was >50 $\mu\text{g}/\text{ml}$, and it demonstrated cross-resistance to oleandomycin, rosaramicin, tylosin, and vernamycin B. The high-level resistant *L. pneumophila* variant had a highly significant increased zone size when tested with clindamycin compared with the erythromycin-susceptible parent strain and in contrast to the high-level resistant *L. micdadei* strain, which demonstrated no increase in zone size with clindamycin. This finding was verified by determining that the clindamycin MICs of the high-level resistant *L. pneumophila* variant and its parent were 4.0 and 11.3 $\mu\text{g}/\text{ml}$, respectively, compared with 32 and 16 $\mu\text{g}/\text{ml}$ for the resistant and parent *L. micdadei* strains, respectively.

All of the MLS antibiotics have a common ribosomal binding site (15). That the high-level resistant *L. pneumophila* variant demonstrated increased susceptibility to clindamycin and decreased susceptibility to the remaining MLS agents indicates that the binding sites for the MLS antibiotics, although overlapping, are not identical. Thus, the high-level *L. pneumophila* and *L. micdadei* resistant variants could have undergone different mutations in synthesis of one

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TABLE 1. Cross-resistance phenotypes to macrolide antibiotics, vernamycin B, and clindamycin of the *Legionella* spp. parent strains and variants isolated from BCYE agar containing various concentrations of erythromycin

Strain	No. tested	Selective concn of erythromycin ($\mu\text{g/ml}$)	Erythromycin MIC ($\mu\text{g/ml}$)	Coresistance phenotype ^a				
				Oleandomycin	Rosaramicin	Tylosin	Vernamycin B	Clindamycin
<i>L. micdadei</i>								
Parent	1		1.4	20	44	23	28	9
Low level resistant	9	2.5	2 to 8	++	—	+	+	—
Intermediate	9	5.0	5 to 32	++	—	—	++	—
Level resistant	1	5.0	8	++	—	—	—	—
High level resistant	1	50	>100	++	++	++	++ ^b	—
<i>L. pneumophila</i>								
Parent	1		0.2	32	51	31	39	12
Low level resistant	5	2.5	3 to 4	++	—	+	++	—
High level resistant	1	10	>50	++	+	++	+	—

^a Coresistance was assayed by disk susceptibility testing. For the parent strains the mean disk diffusion zone size (millimeters) is given. For the resistant variants + indicates a decreased zone size (decreased susceptibility) for the variant compared with the parent that is significant at the ≤ 0.05 level; and ++ indicates a difference that is significant at the ≤ 0.01 level; — indicates no change in susceptibility; and — indicates an increased zone size (hypersusceptibility) for the variant compared with the parent that is significant at the ≤ 0.001 level.

^b Variable; see the text.

of the ribosomal components that constitute the common portion of the MLS binding site such that for the *L. micdadei* variant the binding of all MLS antibiotics was markedly impaired, whereas for *L. pneumophila* ribosomal binding of the macrolides and vernamycin B was modestly reduced and the affinity for clindamycin was actually enhanced. Such alteration in the macrolide binding site has been found for erythromycin-resistant mutants of *Escherichia coli* in which the 50S ribosomal protein L4 is altered, and binding of the antibiotic to isolated ribosomes is markedly reduced (9, 10, 16). However, in other resistant *E. coli* mutants the affinity of erythromycin for the ribosome is unchanged (9, 10). Inducible resistance to erythromycin in *Staphylococcus aureus* has been associated with modification of the 23S rRNA by the introduction of two dimethyladenine residues (6). A concentration of 10^{-7} M erythromycin induces resistance to concentrations as high as 10^{-4} M within a few minutes. We examined both *L. micdadei* and *L. pneumophila* in this manner and did not find inducible resistance. However, since constitutive resistance to erythromycin in one strain of *S. aureus* was found to be associated with altered rRNA (6), this mechanism for erythromycin resistance in the *Legionella* spp. variants is not eliminated by finding that resistance was not inducible.

The ease with which erythromycin-resistant *Legionella* spp. variants were obtained on exposure to modest concentrations of the antibiotic raises the possibility that resistant strains could be selected during therapy of the individual patient with legionellosis. Growth of the resistant variants during the first passage on erythromycin-free medium was as rapid as that of the parent strains, but the high-level erythromycin-resistant variants had to be passed twice on BCYE agar and once through buffered yeast extract broth before the growth in buffered yeast extract broth was sufficient to examine a formal growth curve. When examined by the methods previously described (2), the growth curves of the thrice-passaged, high-level erythromycin-resistant variants of *L. micdadei* and *L. pneumophila* in buffered yeast extract broth over 48 h were virtually identical to the growth curves of the respective parent strains. Despite regaining an equivalent growth rate in vitro, however, the high-level erythromycin-resistant *L. micdadei* variant was found to be less pathogenic for eggs than its parent strain, with a 50% lethal

dose of 3,230 CFU/egg, compared with <21 CFU/egg for the parent (8). This implies either that the multiplication of the resistant variant in vivo was still slower than the parent strain, or that the genotypic change undergone by the resistant variant lead to decreased pathogenicity by some other mechanism.

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