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In Vitro Synergy Testing of Macrolide-Quinolone Combinations against 41 Clinical Isolates of *Legionella*

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Combination antimicrobial therapy against *Legionella* species has not been well studied. Several quinolones have activity against *Legionella* strains, which prompted this in vitro search for a synergistic combination with the macrolides. By a checkerboard assay, erythromycin, clarithromycin, and azithromycin, each in combination with ciprofloxacin and levofloxacin, were tested for synergy against 46 isolates of *Legionella*. The agar dilution method was employed using buffered charcoal-yeast extract media. A final inoculum of 10^4 CFU per spot was prepared from 24-h growth of each isolate. Plates were incubated at 35°C for 48 h. Synergy, partial synergy, additive effect, or indifference was observed for all combinations of antibiotics tested. There was no antagonism observed. Synergy occurred to a significantly greater extent for the clarithromycin-levofloxacin ($P = 0.0001$) and azithromycin-levofloxacin ($P = 0.003$) combinations versus erythromycin-levofloxacin. The azithromycin-ciprofloxacin combination demonstrated significantly greater synergy than did either erythromycin-ciprofloxacin ($P = 0.003$) or clarithromycin-ciprofloxacin ($P = 0.001$). The newer macrolides clarithromycin and azithromycin may be more active in combination with a fluoroquinolone than is erythromycin.

Legionella pneumonia remains a difficult infection to cure despite a large armamentarium of antimicrobial agents which demonstrate activity against *Legionella* species. Monotherapy with erythromycin and combination therapy with erythromycin and rifampin have been the accepted treatments of choice for *Legionella* infections. Anecdotal reports of fluoroquinolone or other macrolide use in cases of erythromycin failure have been published, with various success rates for the alternative agents (9, 13, 14). Very few data comparing various drug regimens either in vitro or in vivo are available. In vitro data support the activity of quinolones (ciprofloxacin, perfloxacin, fleroxacin, temafloxacin, ofloxacin, lomefloxacin, norfloxacin, and sparfloxacin) as well as macrolides (roxithromycin, rosaramicin, josamycin, pristinamycin, midecamycin, spiramycin, azithromycin, and clarithromycin) against *Legionella* species; however, little effort has been made to investigate the combined effect of these two antibiotic classes against these organisms (2, 3, 5–7, 10).

We examined the in vitro activities of erythromycin, clarithromycin, and azithromycin each in combination with ciprofloxacin and levofloxacin, the *l*-isomer (active isomer) of ofloxacin, against 41 clinical isolates of *Legionella* species to determine if these antibiotics have enhanced activity when used together.

(Part of this research was presented at the Second International Conference on the Macrolides, Azalides and Streptogramins, February 1994, Venice, Italy [abstract 137].)

MATERIALS AND METHODS

Bacterial strains. Forty-one clinical strains and five American Type Culture Collection strains representing seven *Legionella* species were tested (Table 1). The organisms were obtained from Abbott Laboratories (Chicago, Ill.), the Centers for Disease Control and Prevention (Atlanta, Ga.), the University of Illinois Hospital Microbiology Laboratory (Chicago, Ill.), Northwestern University Hospital Microbiology Laboratory (Chicago, Ill.), and the American Type Culture Collection (Rockville, Md.). All organisms were stored at −70°C in prereduced anaerobically sterilized milk (Carr Scarborough Microbiologicals, Stone Mountain, Ga.) and underwent three subcultures prior to susceptibility testing. Organisms were not exposed to antibiotic-containing media prior to testing.

Media. The *Legionella* organisms were maintained on buffered charcoal-yeast extract (BCYE) agar (Micro Diagnostics, Lombard, Ill.). The antibiotics were diluted in BCYE agar (Oxoid-Unipath, Ogdensburg, N.Y.) for susceptibility and synergy testing. All antibiotic-containing media were prepared 1 day prior to testing and stored in plastic bags at 4 to 8°C. The total volume in each petri plate was 20 ml. The plates for synergy testing contained 1.0 ml of each antibiotic to be tested and 18 ml of BCYE agar. The plates for the MIC determination contained 1.0 ml of antibiotic, 1.0 ml of either sterile distilled water (for levofloxacin and ciprofloxacin) or sterile potassium phosphate buffer (pH 6.5) (for erythromycin, clarithromycin, and azithromycin), and 18 ml of BCYE agar.

Antibiotics. Erythromycin powder (lot 140988-AX) and clarithromycin powder (lot 252521-AX) were supplied by Abbott Laboratories, azithromycin powder (lot 64QCS-12) was supplied by Pfizer Laboratories (New York, N.Y.), levofloxacin powder (lot 317-76-H) was supplied by Ortho Laboratories (Raritan, N.J.), and ciprofloxacin powder (lot F-1) was obtained from the USP (Rockville, Md.). Stock solutions of 1,000 μg/ml were prepared according to the manufacturers' recommendations, with methanol as the solvent and potassium phosphate buffer (pH 6.5) as the diluent for erythromycin, clarithromycin, and azithromycin and with sterile distilled water as the solvent and diluent for ciprofloxacin and levofloxacin. Stock solutions were stored in polyethylene vials at −70°C until the day of use. After the stock solutions were thawed, serial twofold dilutions of the antibiotics were prepared with potassium phosphate buffer (pH 6.5) for the macrolides and sterile distilled water for the quinolones. Working concentrations ranging from 1.25 to 40.0 μg/ml were prepared to obtain final antibiotic concentrations of 0.0625 to 2.0 μg/ml for the checkerboard assay.

Bacterial inoculum. All organisms were grown overnight (24 h) on BCYE agar plates at 35°C in room air and harvested during the stationary growth phase. A direct suspension of organisms was prepared in 5 ml of sterile distilled water. The turbidity of the suspension was adjusted to match a 0.5 McFarland standard with a spectrophotometer (Spectronic 21; Milton Roy Co., Rochester, N.Y.) at 625 nm, which corresponds to 2.4×10^8 CFU/ml. The suspension was further diluted 1:100 with sterile distilled water.

Checkerboard procedure. The checkerboard procedure was performed using the agar dilution susceptibility procedure (4). A replicator device (Craft Machine Inc., Chester, Pa.) was used to inoculate approximately 8 μl of bacterial inoculum onto the BCYE agar plates, resulting in an inoculum of approximately 10^4 CFU per spot. BCYE agar plates without antibiotics were used as a growth control, while blood agar plates (Micro Diagnostics Inc.) were included as a control measure to ensure purity of the bacterial inoculum. All organisms were tested in two batches, with both sets conducted on the same day. Each plate was inoculated with 23 different *Legionella* organisms and one control strain, *Staphylococcus aureus* ATCC 14154. All tests were performed in duplicate, and all plates were incubated in room air at 35°C for 48 h.

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TABLE 1. MICs of erythromycin, clarithromycin, azithromycin, ciprofloxacin, and levofloxacin for 41 clinical isolates of *Legionella*

Legionella species (no. of strains)	MIC(s) (μg/ml) of:				
	Erythromycin	Clarithromycin	Azithromycin	Ciprofloxacin	Levofloxacin
L. pneumophila (34)	0.25–1 (0.5)[a]	0.125–0.5 (0.25)	0.125–2 (0.5)	1–2 (1.0)	0.5–1 (0.5)
L. micdadei (1)	0.25	0.25	0.5	0.5	0.25
L. dumoffii (1)	1	0.5	2	1	0.5
L. erythra (1)	1	1	2	1	0.5
L. bozemanii (2)	0.25, 1	0.25	0.5, 1	0.5, 1	0.25, 1
L. longbeachae (2)	0.5, 1	0.5	1	0.5, 1	0.25, 0.5

[a] Values for *L. pneumophila* report the MIC range and, in parentheses, the MIC at which 50% of the strains tested were inhibited.

Endpoint determination. The MIC was read as the lowest concentration of antimicrobial agent(s) showing no visible growth or only a faint haze. MICs were determined for each agent individually and in combination.

Data analysis. Synergy was determined by calculating the fractional inhibitory concentration (FIC) index (4) as follows: FIC index = $FIC_A + FIC_B = [A]/MIC_A + [B]/MIC_B$, where $[A]$ is the concentration of drug A that is the lowest inhibitory concentration, MIC_A is the MIC of drug A for the organism, FIC_A is the FIC of drug A, and $[B]$, MIC_B, and FIC_B are defined in the same fashion for drug B. Results for synergy testing were defined according to guidelines established by the American Society for Microbiology (1) so that a given FIC index was interpreted as follows: ≤0.5, synergy; >0.5 to 0.75, partial synergy; >0.76 to 1.0, additive effect; >1.0 to 4.0, indifference; and >4.0, antagonism. Chi-square analysis was performed to determine differing rates of synergy for the various combinations.

RESULTS

All *Legionella* strains grew well on antibiotic-free BCYE agar after 48 h of incubation. No contaminants were noted. MICs for each organism of all antibiotics tested are listed in Table 1. MICs for the control strains were within expected limits. Results of the checkerboard tests are listed in Tables 2 and 3. The FIC indices for all of the combinations tested ranged from 0.375 to 2.0; synergy, partial synergy, additive effect, or indifference was observed for all the combinations of antibiotics tested. No antagonism was identified in the range of concentrations tested. Distribution of FIC indices for the combinations of antibiotics against *Legionella pneumophila* and non-*pneumophila Legionella* species are presented in Tables 2 and 3.

DISCUSSION

We examined the effects of various macrolide-quinolone combinations on several different species of *Legionella* clinical isolates to determine possible antimicrobial synergy. BCYE agar was chosen for the checkerboard analysis because of the difficulty in growing and maintaining *Legionella* strains without charcoal media, especially non-*pneumophila* species (11). Although charcoal is reported to inactivate various antibiotics, we felt that the MICs obtained would provide the worst possible representation of antimicrobial activity (2). All MICs determined in this experiment are easily achievable in serum with recommended doses of the antibiotics tested. Our findings demonstrate that there may be a benefit to combinations of macrolides and fluoroquinolones for *Legionella* species. Although synergy was observed for some combinations, partial synergy, additive effect, or indifference was most commonly observed for all combinations of antimicrobial agents against all strains tested.

Predominantly additive effects (FIC index, >0.76 to 1) were found with the combination of the quinolones and erythromycin against *L. pneumophila* species. The combination of clarithromycin with ciprofloxacin also proved to be only additive against these species. However, since clarithromycin has an active 14-hydroxy metabolite in vivo which may be responsible for a significant portion of the compound's activity against gram-negative organisms, this could account for the lack of synergy in our results. Our laboratory and Jones et al. have found anti-*Legionella* activity in the 14-hydroxy clarithromycin metabolite (8, 12). Thus, the in vitro effect of clarithromycin-metabolite combination alone and combined with the quinolones against *Legionella* species should be further investigated in vivo.

Synergy or partial synergy was evident in the majority of the *L. pneumophila* isolates tested with the combinations of clarithromycin-levofloxacin and azithromycin-levofloxacin. Synergy occurred to a significantly greater extent with the clarithromycin-levofloxacin ($P = 0.0001$) and azithromycin-levofloxacin ($P = 0.003$) combinations than with erythromycin-levofloxacin. The azithromycin-ciprofloxacin combination demonstrated significantly greater synergy than either erythromycin-ciprofloxacin ($P = 0.003$) or clarithromycin-ciprofloxacin ($P = 0.001$).

Although the data are preliminary with the non-*pneumophila Legionella* species, the combination of levofloxacin with any of the macrolides appears to have more activity than those with ciprofloxacin.

On the basis of these results, we believe that further studies are warranted to determine the in vivo effect of macrolide-quinolone combinations on *Legionella* infections.

TABLE 2. FIC index of drug combinations against 34 clinical isolates of *L. pneumophila*

Drug combination	No. of isolates for which FIC index is:						
	0.375	0.5	0.563	0.625	0.75	1.0	2.0
Erythromycin-ciprofloxacin					5	2	27
Erythromycin-levofloxacin			2	1	1	10	20
Clarithromycin-ciprofloxacin						4	30
Clarithromycin-levofloxacin				7	12	10	5
Azithromycin-ciprofloxacin	2		2	5	7	6	12
Azithromycin-levofloxacin		5		7	9	5	8

TABLE 3. FIC index of drug combinations against seven clinical isolates of non-*pneumophila Legionella*

Legionella species (no. of strains)	FIC index of:					
	Erythromycin-ciprofloxacin	Erythromycin-levofloxacin	Clarithromycin-ciprofloxacin	Clarithromycin-levofloxacin	Azithromycin-ciprofloxacin	Azithromycin-levofloxacin
L. micdadei (1)	2	2	2	1	0.5	1
L. dumoffii (1)	1	2	2	0.75	0.563	1
L. erythra (1)	0.75	0.75	2	0.625	2	1
L. bozemanii (2)	1, 2	0.625, 2	2	0.75, 2	1, 2	0.75, 2
L. longbeachae (2)	0.625, 2	0.625, 2	0.625, 2	0.75, 2	0.75, 2	0.75, 1

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