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Activities of Levofloxacin, Ofloxacin, and Ciprofloxacin, Alone and in Combination with Amikacin, against Acinetobacters as Determined by Checkerboard and Time-Kill Studies

SARALEE. BAJAKSOUZIAN,[1] MELISSA A. VISALLI,[2] MICHAEL R. JACOBS,[1] AND PETER C. APPELBAUM[2]*

Department of Pathology (Clinical Microbiology), Case Western Reserve University, Cleveland, Ohio 44106,[1] and Department of Pathology, Hershey Medical Center, Hershey, Pennsylvania 17033[2]

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A total of 101 *Acinetobacter* genospecies (77 *Acinetobacter baumannii* strains and 24 non-*A. baumannii* strains) were tested for their susceptibilities to levofloxacin, ofloxacin, and ciprofloxacin and for synergy between the quinolones and amikacin by checkerboard titration and time-kill analyses. The MICs at which 50% of the isolates are inhibited (MIC_{50})/MIC_{90}s for the 101 strains were as follows (in micrograms per milliliter): levofloxacin, 0.25/16.0; ofloxacin, 0.5/32.0; ciprofloxacin, 0.25/>64.0; and amikacin, 1.0/>32.0. At empiric breakpoints of ≤2.0 μg/ml, 61% of the strains were susceptible to all three quinolones. At a breakpoint of ≤16.0 μg/ml, 84% of the strains were susceptible to amikacin. Checkerboard titrations yielded synergistic fractional inhibitory concentration (FIC) indices (≤0.5) for one strain with levofloxacin and amikacin and for two strains with ofloxacin and amikacin. Indices of >0.5 to 1.0 were seen for 57, 54, and 55 strains with levofloxacin plus amikacin, ofloxacin plus amikacin, and ciprofloxacin plus amikacin, respectively, and indices of >1.0 in 43, 45, and 46 strains, respectively, were found with the above three combinations. No strains yielded antagonistic FIC indices (>4.0). Most FIC results of >1.0 occurred in strains for which the quinolone MICs were >2.0 μg/ml and for which the amikacin MICs were ≥32.0 μg/ml. By contrast, synergy (defined as ≥2 $\log_{10}$ decrease compared to the more active compound alone by time-kill analysis) was found in all seven strains tested for which the quinolone MICs were ≤2.0 μg/ml. For eight other strains for which the quinolone MICs were >2.0 μg/ml as determined by time-kill analysis, quinolone and amikacin concentrations in combination were usually too high to permit clinical use. Time-kill analysis was found to be more sensitive in detecting synergy than was the checkerboard method.

Gram-negative, nonfermentative rods are increasingly implicated as causative agents in human disease and are acquired through contact with environmental strains, as well as nosocomially. Although *Pseudomonas aeruginosa* is the nonfermenter most commonly encountered clinically, other gram-negative, nonfermentative rods are being recovered from debilitated or immunosuppressed patients with increasing frequency (29). Of other nonfermenters, *Acinetobacter* species, especially *Acinetobacter baumannii*, have become important pathogens in debilitated patients, especially in intensive care units. The intrinsic resistance of *A. baumannii* to many β-lactam and non-β-lactam antibiotics results in therapeutic problems (6, 17, 18, 22–25, 29).

Levofloxacin, the *l*-isomer of ofloxacin, has been shown to yield MICs which are one or two dilutions lower than those of ofloxacin and ciprofloxacin (12, 14, 21, 27). In a previous study, we have shown that levofloxacin yielded a MIC at which 50% of the isolates are inhibited (MIC_{50}) of 0.25 μg/ml and a MIC_{90} of 8.0 μg/ml against 72 *Acinetobacter* genospecies (26). Using the time-kill method, Decre and coworkers have demonstrated synergistic activity between levofloxacin and amikacin for *Acinetobacter* strains for which the levofloxacin MICs are ≤1.0 μg/ml (11). We have extended the latter study by using checkerboard as well as time-kill studies to examine the susceptibilities of 101 *Acinetobacter* strains to levofloxacin, ofloxacin, and ciprofloxacin, alone and in combination with amikacin.

* Corresponding author. Mailing address: Department of Pathology, Hershey Medical Center, P.O. Box 850, Hershey, PA 17033. Phone: (717) 531-5113. Fax: (717) 531-7953. E-mail: pappelba@psuhmc.hmc.psu.edu.

MATERIALS AND METHODS

Bacteria. All strains were clinical cultures isolated within the past 4 years. The strains were identified as *A. baumannii* and non-*A. baumannii* (29) by the MIDI (Newark, Del.) gas chromatographic system. The strains were frozen at −70°C in double-strength litmus milk (Difco Laboratories, Detroit, Mich.) prior to testing.

Antimicrobials. Antibiotic powders were obtained as follows: levofloxacin and ofloxacin were from Hoechst-Marion-Roussel, Paris, France, ciprofloxacin was from Bayer Corporation, West Haven, Conn., and amikacin was from Sigma Chemical Co., St. Louis, Mo.

MIC determinations and synergy testing. MIC determinations and synergy testing were performed for 101 strains by the checkerboard method in microtiter trays with cation-supplemented Mueller-Hinton broth (Difco) (13). Levofloxacin, ofloxacin, and amikacin were tested at concentrations of 0.06 to 64.0 μg/ml, and amikacin was tested at concentrations of 0.5 to 32.0 μg/ml. The trays were prepared with a 96-channel dispenser and stored at −70°C until use. Quinolones were dispensed alone in the first row, and amikacin was dispensed in the first column. Inocula were prepared by suspending growth from blood agar plates in sterile saline to a density of a 0.5 McFarland standard, and were diluted 1:10 to produce a final inoculum of 5×10^5 CFU/ml with a multipoint inoculator. The trays were incubated aerobically overnight. Standard quality control strains were included with each run. Fractional inhibitory concentrations (FICs) were calculated as the MIC of drug A or B in combination divided by the MIC of drug A or B alone, and the FIC index was obtained by adding the FICs. FIC indices were interpreted as synergistic when values were ≤0.5, as additive or indifferent when values were >0.5 to 4.0, and as antagonistic when values were >4.0 (13).

Time-kill determinations. Fifteen *Acinetobacter* strains were tested. Quinolones and amikacin were tested alone and in combination. In each case, concentrations of as much as four dilutions higher and four dilutions lower than the MICs were tested. Viability counts were performed at 0, 6, 12, and 24 h. Drug carryover was minimized by dilution as described previously (9, 26). Synergy was defined as a decrease of ≥2 $\log_{10}$ in viability count of the combination at 24 h compared to that with the more active of the two compounds alone (9).

The potential for drug carryover to produce falsely low viability counts was

TABLE 1. Susceptibilities of *Acinetobacter* species to individual agents

Acinetobacter (*n*) and agent	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Susceptible[a]
A. baumannii (77)				
Levofloxacin	0.03–32.0	0.25	16.0	56
Ofloxacin	0.06–64.0	0.5	64.0	54
Ciprofloxacin	0.016–>64.0	0.5	>64.0	54
Amikacin	0.125–>32.0	2.0	>32.0	74
Other *Acinetobacter* genospecies (24)				
Levofloxacin	0.008–0.125	0.06	0.125	100
Ofloxacin	0.016–0.25	0.125	0.25	100
Ciprofloxacin	0.008–0.25	0.06	0.25	100
Amikacin	0.125–32.0	1.0	16.0	90

[a] Breakpoints: quinolones, ≤2 μg/ml; amikacin, ≤16 μg/ml.

minimized by dilution of inocula and plating of small volumes of inocula (100 μl) onto plates containing 20 ml of medium as previously described (4, 9, 26). In addition, the following experiment was carried out to determine if any drug carryover effect could influence viability counts. The highest concentration of each agent was prepared in the broth used for time-kill studies; 100 μl was then placed on blood agar plates, as well as 100-μl volumes of serial 10-fold dilutions of the antibiotic-containing broth. The solutions were then allowed to be absorbed into the agar medium for 20 min. Two organism suspensions were prepared, with strains 1 and 2 used in time-kill experiments (see Table 4), at organism densities of 10^3 and 10^5 CFU/ml. These inocula, as well as serial 10-fold dilutions thereof, were then plated onto the dried blood agar plates containing antibiotics (see above) in 100-μl volumes, as well as onto antibiotic-free blood plates. The plates were incubated overnight, and viability counts were determined. These experiments were performed in duplicate.

RESULTS

The results of MIC testing of the 101 strains are presented in Table 1. The MIC_{50}s/MIC_{90}s (in micrograms per milliliter) for the strains were as follows: levofloxacin, 0.25/16.0; ofloxacin, 0.5/32.0; ciprofloxacin, 0.25/>64.0; and amikacin, 1.0/>32.0. At empiric quinolone breakpoints of ≤2.0 μg/ml, 61% of the strains were susceptible to all three quinolones. At a breakpoint of ≤16.0 μg/ml, 84% of the strains were susceptible to amikacin. Checkerboard titrations (Tables 2 and 3) yielded synergistic FIC indices (≤0.5) for one strain with levofloxacin and amikacin and for two strains with ofloxacin and amikacin. FIC indices of >0.5 to 1.0 were seen for 57, 54, and 55 strains with amikacin combined with levofloxacin, ofloxacin, and ciprofloxacin, respectively, and FIC indices of >1.0 were seen for 43, 45, and 46 strains, respectively. Most cases of FIC indices of >1.0 occurred in strains for which the quinolone MICs were >2.0 μg/ml and for which the amikacin MICs were ≥32.0 μg/ml (Tables 2 and 3). No strains with antagonistic FIC indices (>4.0) were observed.

No difference in viability counts were detected between antibiotic-treated and untreated control plates. The results of time-kill studies, compared to those of checkerboard titration analysis of the 15 strains tested, are presented in Table 4. As can be seen, all seven strains for which the quinolone MICs were ≤2.0 μg/ml yielded synergy when quinolones were combined with amikacin. Levofloxacin yielded the lowest MICs alone and in combination. By contrast, in the eight strains for which quinolone MICs were >2.0 μg/ml, the quinolone concentrations required for synergy were too high to permit clinical use (19, 28); a similar phenomenon was observed in most cases for the three strains for which the amikacin MICs were ≥32.0 μg/ml.

TABLE 2. Results of checkerboard titration studies of 66 strains for which the levofloxacin MICs were at ≤2.0 μg/ml[a]

FIC index	No. of strains tested (%) with:		
	Levofloxacin + amikacin	Ofloxacin + amikacin	Ciprofloxacin + amikacin
≤0.5	1 (1)	2 (3)	0
>0.5–1.0	50 (76)	48 (73)	50 (76)
1–2	15 (23)	16 (24)	16 (24)

[a] These include 42 *A. baumannii* strains and 24 non-*A. baumannii* strains.

TABLE 3. Results of checkerboard titration studies of 35 *A. baumannii* strains for which the levofloxacin MICs were ≥4.0 μg/ml[a]

FIC index	No. of strains tested (%)		
	Levofloxacin + amikacin	Ofloxacin + amikacin	Ciprofloxacin + amikacin
≤0.5	0	0	0
>0.5–1.0	7 (20)	6 (17)	5 (14)
1–2	28 (80)	29 (83)	30 (86)

DISCUSSION

The results of this study show a bimodal distribution of MICs of quinolones against *Acinetobacter* strains, with levofloxacin yielding lower MICs than ofloxacin and ciprofloxacin. This has been reported previously, both by us (26) and by other workers (11). At a breakpoint of ≤2.0 μg/ml (19), 61% of the strains were susceptible to all three quinolones. Checkerboard titrations did not yield significant synergy between any of the three quinolones and amikacin. However, time-kill testing yielded synergy for all strains for which the quinolone MICs were ≤2.0 μg/ml. Levofloxacin breakpoints of ≤2.0 μg/ml (susceptible), 4 μg/ml (intermediate), and ≥8.0 μg/ml (resistant) have recently been approved for members of the family *Enterobacteriaceae*, and these breakpoints also apply to *Acinetobacter* species and other gram-negative nonfermenters (19).

Comparison of *Acinetobacter* susceptibility patterns has been complicated by recent taxonomic changes (29). Because the classification of *Acinetobacter* is in a state of flux, we elected to divide organisms into *A. baumannii* (the commonest genospecies encountered in clinical specimens) and non-*A. baumannii* strains, which currently comprise 18 named and unnamed genospecies which are difficult to differentiate by routine procedures (29).

Although some workers have found *Acinetobacter* strains to be susceptible to quinolones (1–3, 5–8, 10, 14, 15, 20–22, 27), others have reported these strains (especially *A. baumannii*) to be increasingly resistant to quinolone and nonquinolone antibiotics in recent years (12, 16, 23–25, 28, 30). A recent report from Singapore (where *Acinetobacter* is the commonest pathogen in intensive care units) has documented higher quinolone MICs than those that are encountered in the United States (18).

We believe that the problem of drug carryover was adequately addressed. Spreading of 100 μl of undiluted broth onto a plate containing 20 ml of medium would dilute the drug 1:200; further 10-fold dilutions would dilute drugs 1:2,000, 1:20,000, etc. With the concentrations of drugs tested, only undiluted inocula would have had any potential for drug carryover, and only low counts (<1,000 CFU/ml) would be likely to be affected. Additionally, experiments on plates with and without antibiotics failed to reveal significant differences in

TABLE 4. Comparison of checkerboard and time-kill studies[a]

Strain no.	Amikacin MIC (μg/ml)	Levofloxacin MIC (μg/ml)	Ofloxacin MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	Value with levofloxacin + amikacin		Value with ofloxacin + amikacin		Value with ciprofloxacin + Amikacin	
					C	T[b]	C	T[b]	C	T[b]
1	4	0.06	0.125	0.25	Ad	0.06/0.5	Ad	0.06/0.25	Ad	0.06/0.5
2	4	0.125	0.125	0.5	Ad	0.06/0.5	Ad	0.125/0.5	Ad	0.25/2
3	2	0.25	0.25	0.25	Ad	0.03/1	Ad	0.06/1	Ad	0.25/2
4	4	0.125	0.25	0.25	Ad	0.06/2	Ad	0.25/2	Ad	0.12/4
5	4	0.125	0.5	0.5	Ad	0.06/1	Ad	0.12/2	Ad	0.5/2
6	4	0.125	0.25	0.25	Ad	0.06/2	Ad	0.25/2	Ad	0.06/2
7	8	0.125	0.125	0.125	Ad	0.12/2	Ad	0.12/4	Ad	0.12/1
8	16	4	8	8	Ad	4/16	Ad	8/16	Ad	8/16
9	1	8	16	>64	Ad	4/1	Ad	16/1	Ad	128/2
10	1	8	16	>64	Ad	8/1	Ad	16/1	Ad	128/2
11	32	16	32	>64	Ad	16/32	Ad	32/32	Ad	64/32
12	8	8	32	>64	Ad	8/1	Ad	16/1	Ad	128/2
13	64	8	32	64	Ad	4/16	Ad	8/16	Ad	16/32
14	64	8	16	16	Ad	2/8	Ad	8/16	Ad	8/16
15	2	16	32	>64	Ad	16/2	Ad	32/2	Ad	256/4

[a] C, checkerboard analysis; T, time-kill assay; Ad, additive or indifferent.
[b] Values are the lowest concentrations (in micrograms per milliliter) of each compound in combination that yielded synergy.

colony counts. Therefore, we feel that drug carryover was not a confounding factor in data generation.

Our study emphasizes the difference between checkerboard and time-kill methods in the detection of synergy among *Acinetobacter* strains. The same discrepancy has been encountered in *P. aeruginosa* (9) and *Streptococcus pneumoniae* (4). Methodologies and definitions of synergy and antagonism differ, and there is a need for re-evaluation of methods to detect synergy (13). It should be noted that checkerboard titration tests bacteriostatic activity only, while time-kill studies test both bacteriostatic and bactericidal activities. However, in strains for which the quinolone MICs are ≤2.0 μg/ml, subinhibitory concentrations of at least one of the two compounds are achievable clinically. In cases in which the quinolone MICs were ≥4.0 μg/ml, the quinolone MICs in the combination were usually not achievable clinically; the same applied in most cases for the three strains for which the amikacin MICs were ≥32.0 μg/ml.

Time-kill results in the current study are similar to those reported by Decre and coworkers (11). The results of this (11) and our study indicate that levofloxacin may represent a significant alternative in the therapy of *Acinetobacter* infections, both alone and in combination with amikacin. However, each isolate should be carefully tested for in vitro susceptibility to the quinolone alone before single or combination therapy is instituted. Clinical studies will be necessary to validate these hypotheses.

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