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Lack of Effective Bactericidal Activity of New Quinolones against *Brucella* spp.

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The in vitro activities of six fluoroquinolones against 43 *Brucella* spp. were compared by testing three different inocula at two medium pH values. The influence of the test conditions was moderate. The activities of all quinolones were lower at pH 5 and with a high inoculum size. Results indicate the lack of effective bactericidal activity of quinolones against most strains of *Brucella* spp., particularly *B. abortus*.

Although brucellosis is primarily a disease of domestic animals, it is a public health problem in many countries, especially the Mediterranean countries. The treatment of brucellosis requires combined regimens of antibiotics and is conditioned by the fact that brucellae are intracellular pathogens; thus, agents with a good capacity to penetrate macrophages are required for successful treatment. The regimen recommended for the treatment of brucellosis is a combination of oral tetracycline (or doxycycline) plus intramuscular streptomycin. Another regimen used is doxycycline plus rifampin (1, 13). Each of these regimens has disadvantages (13). The necessity of combined treatment, the length of treatment, and the proportion of therapeutic failures with some regimens oblige us to look for new drugs for the treatment of brucellosis. Fluoroquinolones, as a class, exhibit a broad spectrum of antibacterial activity (18). Their oral bioavailabilities, high tissue concentrations, evidence of intracellular penetration (fluoroquinolones appear to achieve intracellular concentrations in phagocytic cells significantly in excess of extracellular concentrations) (4–6, 14), and in vitro activities against *Brucella* spp. (2, 3, 7, 9, 10, 12, 13) make these antimicrobial agents attractive for use against infections caused by intracellular bacteria such as *Brucella* spp.

The purpose of the present study was to determine the effect of the pH of the medium and inoculum size on the MICs and MBCs of ciprofloxacin, ofloxacin, lomefloxacin, temafloxacin, fleroxacin, and sparfloxacin against *Brucella* spp. Here, we report the results of the effect of inoculum density (10^3, 10^4, and 10^6 CFU per spot) and pH of the medium (pHs 7 and 5; pH of the phagolysosome [16]) on the MICs (assessed by the agar dilution method) of these fluoroquinolones for *B. melitensis* and *B. abortus* and the effect of pH of the medium on the MICs (assessed by the broth macrodilution method) and MBCs of the same antimicrobial agents for 21 strains of *B. melitensis* and 4 reference strains of *B. abortus*, with the objective of determining the bactericidal capacity of these antibiotics, since this is the principal factor for the treatment of brucellosis.

(This work was presented in part at the 30th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, Ga., 21 to 24 October 1990 [8].)

The following antibiotics were kindly supplied by their manufacturers: ciprofloxacin (Bayer), ofloxacin (Hoechst), lomefloxacin (Searle), temafloxacin (Abbott Laboratories Ltd.), fleroxacin (Roche), and sparfloxacin (Rhone-Poulenc). A total of 33 recent clinical isolates of *B. melitensis*, the reference strain of the biotype 1 strain ATCC 23456, and 9 reference strains of *B. abortus* (ATCC 23448 biotype 1, ATCC 23449 biotype 2, ATCC 23450 biotype 3, ATCC 23451 biotype 4, ATCC 23452 biotype 5, ATCC 23453 biotype 6, ATCC 23455 biotype 9, and NCTC 8038 biotype 1 and NCTC 11363 biotype 1) were tested. All clinical strains were isolated from clinical specimens at the Hospital Clínico Universitario of Salamanca, Salamanca, Spain. The organisms were identified by standard methods and were stored frozen at −70°C. The MICs of the antimicrobial agents were determined by the agar dilution method described for *Haemophilus influenzae* susceptibility tests (17). The culture medium was Mueller-Hinton agar (Oxoid Ltd., Basingstoke, England) supplemented with 1% hemoglobin (bioMerieux, Charbonnieres les Bains, France) and 1% PoliViteX (bio Merieux). The antimicrobial stock solutions were prepared at concentrations of 1,280 mg/liter and were stored frozen in small volumes in sterile polypropylene vials at −70°C. Serial twofold dilutions of antimicrobial agents were made in Mueller-Hinton broth supplemented with 1% PoliViteX. The range of concentrations assayed for each antibiotic was 0.008 to 4 mg/liter. In all cases, we considered the directions provided by the drug manufacturers as a part of these general recommendations. *Brucella* inocula were dilutions of 48-h broth cultures which were inoculated from a stock brucella slant that was stored frozen. Standard *Brucella* inocula were carried out by touching the top of four to five colonies of a single type and inoculating them into a tube containing 3.0 ml of brucella broth. These bacterial suspensions were incubated at 35°C for 48 h in an atmosphere of 10% CO_2. The turbidities of these active growth cultures were adjusted spectrophotometrically from a 48-h culture plate, as verified by colony counts, to a turbidity equivalent to that of a no. 0.5 McFarland turbidity standard (10^8 CFU/ml) by adding the same broth. These adjusted suspensions were diluted 1/10 in the same sterile broth to obtain inoculum concentrations of 10^7 CFU/ml. The final inoculum (standard inoculum) was 10^4 CFU per spot, since the inoculum replicator used plates with 10^{-3} ml on the agar surface. A Steers replicator was used to inoculate a final inoculum of 10^4 CFU per spot onto the surfaces of the antibiotic-containing agar plates. The agar plates were incubated at 35°C for 48 h in an atmosphere of 10% CO_2. Results (MICs) were read and recorded after 48 h of incubation. The control strains tested indicated that the pH was not changed. At the beginning and end of each series of tests with each antimi-

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TABLE 1. Effect of inoculum and pH on MICs of six quinolones for 34 strains of *B. melitensis*

Inoculum (CFU/spot)	Antimicrobial agent	MIC (mg/liter)[a]					
		pH 5			pH 7		
		Range	50%	90%	Range	50%	90%
10^3	Ciprofloxacin	0.5–1	1	1	0.25–0.5	0.5	0.5
	Ofloxacin	1–2	2	2	0.5–1	1	1
	Lomefloxacin	2–4	2	4	1–2	2	2
	Temafloxacin	0.25–2	1	1	0.125–0.5	0.25	0.25
	Fleroxacin	1	1	1	0.5–1	0.5	1
	Sparfloxacin	0.125–0.5	0.25	0.25	0.06–0.25	0.125	0.125
10^4	Ciprofloxacin	0.5–1	1	1	0.25–0.5	0.5	0.5
	Ofloxacin	1–2	2	2	0.5–1	1	1
	Lomefloxacin	2–4	2	4	1–2	2	2
	Temafloxacin	0.25–1	1	1	0.125–0.5	0.25	0.25
	Fleroxacin	1	1	1	0.5–1	0.5	1
	Sparfloxacin	0.25–0.5	0.5	0.5	0.125–0.5	0.25	0.25
10^6	Ciprofloxacin	1–2	2	2	0.5–1	1	1
	Ofloxacin	2–4	2	4	1–2	1	2
	Lomefloxacin	4–≥8	4	≥8	4	4	4
	Temafloxacin	0.5–2	1	1	0.25–0.5	0.25	0.25
	Fleroxacin	1–2	2	2	1	1	1
	Sparfloxacin	0.5–1	0.5	0.5	0.25–0.5	0.25	0.25

[a] MICs are those obtained by the agar dilution method. 50% and 90%, MIC for 50 and 90% of isolates, respectively.

crobial agent tested, supplemented Mueller-Hinton agar plates without antibiotics were also inoculated under the same conditions to indicate possible aerobic contamination and to verify good growth of the strains being tested. All determinations were performed twice. The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited growth; a single colony or a faint haze caused by the inoculum was disregarded.

As a previous condition for determining the in vitro bactericidal activity (MBC), we also studied the in vitro activities of the same six fluoroquinolones against 21 strains of *B. melitensis*, including the reference strain ATCC 23456 (biotype 1), and 4 reference strains of *B. abortus* (ATCC 23449 biotype 2, ATCC 23452 biotype 5, ATCC 23453 biotype 6, ATCC 23455 biotype 9), using the broth macrodilution method. Bactericidal activity (MICs were assessed in liquid medium, and MBCs were 99.9% lethal in solid medium) was determined as described by the National Committee for Clinical Laboratory Standards (15). *Brucella* standard inocula were carried out by touching the top of four to five colonies of a single type and inoculating them into a tube containing 3.0 ml of brucella broth. These bacterial suspensions were incubated at 35°C for 48 h in an atmosphere of 10% CO_2. The turbidities of these active growth cultures were adjusted spectrophotometrically from a 48-h culture plate, as verified by colony counts, to obtain a turbidity comparable to that of a no. 0.5 McFarland turbidity standard by adding the same broth. These adjusted standard cultures were diluted in Mueller-Hinton broth supplemented with 1% PoliViteX to 2×10^4 CFU/ml (spectrophotometric adjustment of the inoculum from a 48-h culture plate verified by colony counts). Equal volumes (1 ml) of diluted cultures were added to each antimicrobial dilution (serial twofold dilutions of antimicrobial agents from 8 to 0.01 mg/liter) in Mueller-Hinton broth supplemented with 1% PoliViteX, leading to a final inoculum of 10^4 CFU/ml in a final volume of 2 ml. The final range of concentrations of fluoroquinolones was from 4 to 0.008 mg/liter, and the final inoculum was 10^4 CFU/ml. The tubes were incubated at 35°C for 48 h in an atmosphere of 10% CO_2. Subcultures were made for confirmation of purity and for quantification of the inoculum size. The MICs in liquid medium were defined as the lowest concentrations of antimicrobial agent that completely inhibited development of visible growth in broth. MBCs were determined by quantitative subculturing of 0.1 ml of broth from the control tube, the first tube containing growth, and from all tubes without visible growth on drug-free Mueller-Hinton agar plates supplemented with hemoglobin and PoliViteX. The plates were incubated at 35°C for 48 h in an atmosphere of 10% CO_2 for colony formation. The MBCs were defined as the lowest drug concentrations that killed ≥99.9% of the initial inoculum; reductions in numbers were assessed by viable counts. All tests were performed twice.

The influences of the pH of the medium and the inoculum size on the in vitro antibacterial activity were also studied. We checked the pH of each batch of supplemented Mueller-Hinton agar after we sterilized it. The pH was adjusted before sterilization by adding 1 N HCl or 1 N NaOH and was measured with a pH meter (PHM 83 Autocal radiometer). To ensure that the pH of the medium was 7 (at 25°C), we measured the pH of the medium after the medium had solidified. Measurements were made with a surface electrode (Tecan) after the supplements were added to agar. The inoculum sizes (10^3, 10^4, and 10^6 CFU per spot) were adjusted spectrophotometrically from a 48-h culture plate, as verified by colony counts.

Table 1 shows the effect of a medium pH of 5 or 7 and a bacterial inoculum of 10^3, 10^4, or 10^6 CFU per spot on the MICs (assessed by the agar dilution method) of six quinolones for 34 strains of *B. melitensis*. Variation of inoculum densities at pHs 7 and 5 did not have a significant effect on the activities of these drugs within the range of inocula tested. The increase in the MICs in terms of changes in inoculum density (from 10^3 to 10^6 CFU per spot) was one dilution. Antibacterial activity was not significantly influenced by this. At pH 7 and within the range of inocula tested,

TABLE 2. Effect of inoculum and pH on MICs of six quinolones for nine reference strains of *B. abortus*

Inoculum (CFU/spot)	Antimicrobial agent	MIC (mg/liter)[a]					
		pH 5			pH 7		
		Range	50%	90%	Range	50%	90%
10^3	Ciprofloxacin	1–2	2	2	0.5–1	1	1
	Ofloxacin	1–2	2	2	0.5–1	1	1
	Lomefloxacin	2–4	2	2	1–2	2	2
	Temafloxacin	4–≥8	≥8	≥8	2–≥8	4	≥8
	Fleroxacin	1	1	1	0.5–1	1	1
	Sparfloxacin	4–≥8	≥8	≥8	2–≥8	4	≥8
10^4	Ciprofloxacin	1–2	2	2	0.5–1	1	1
	Ofloxacin	1–2	2	2	0.5–1	1	1
	Lomefloxacin	2–4	2	2	1–2	2	2
	Temafloxacin	4–≥8	≥8	≥8	2–≥8	4	≥8
	Fleroxacin	1	1	1	1	1	1
	Sparfloxacin	4–≥8	≥8	≥8	2–≥8	4	≥8
10^6	Ciprofloxacin	2–4	4	4	1–2	2	2
	Ofloxacin	2–4	4	4	1–2	2	2
	Lomefloxacin	4–≥8	4	≥8	4	4	4
	Temafloxacin	≥8	≥8	≥8	4–≥8	4	≥8
	Fleroxacin	1–2	2	2	1	1	1
	Sparfloxacin	≥8	≥8	≥8	4–≥8	4	≥8

[a] MICs are those obtained by the agar dilution method. 50% and 90%, MIC for 50 and 90% of isolates, respectively.

the most active quinolones (MIC for 90% of strains tested [MIC_{90}], <1 mg/liter) were sparfloxacin (MIC_{90}, 0.125 mg/liter at 10^3 CFU per spot and 0.25 mg/liter at 10^4 and 10^6 CFU per spot) and temafloxacin (MIC_{90}, 0.25 mg/liter in all inocula tested). The in vitro activities of the same six fluoroquinolones against *B. melitensis* were modified by the pH of the medium. The MICs of all quinolones tested were two to four times higher at pH 5 compared with the MICs obtained at pH 7. At pH 5, sparfloxacin was the most active quinolone (MIC_{90}, 0.25 mg/liter at 10^3 CFU per spot and 0.5 mg/liter at 10^4 and 10^6 CFU per spot). Table 2 shows the effect of medium pH and inoculum size on MICs (assessed by the agar dilution method) of the same six quinolones against *B. abortus*. Variations of the inoculum size and the pHs of the medium did not have a significant effect on the activities of the fluoroquinolones. The activities of temafloxacin and sparfloxacin against *B. abortus* were the lowest among those of the quinolones tested within the range of inocula and pH of the medium tested (MIC_{90} range, ≥8 mg/liter). Fleroxacin was slightly more active than any of the other quinolones tested (MIC_{90} range, 1 to 2 mg/liter). Table 3 shows the effect of medium pH on MICs assessed by the broth dilution method and MBCs of quinolones tested at standard inoculum size (10^4 CFU/ml) against *B. melitensis* and *B. abortus*. As observed by comparison of the data in Tables 1, 2, and 3, the MICs obtained by the broth macrodilution method were at least to two to four times higher than those obtained by the agar dilution method. At pH 7, the MICs for *B. melitensis* were two to four times higher than those observed at the same pH by the agar dilution method. The most active quinolones tested by the broth dilution method were also temafloxacin and sparfloxacin (MIC_{90}s, 1 mg/liter). At pH 5, all MICs were ≥8 mg/liter except for those for two strains of this organism, whose temafloxacin

TABLE 3. Effect of pH on MICs and MBCs of six quinolones for *B. melitensis* and *B. abortus*

Species (no. of isolates tested)	Antimicrobial agent	MIC/MBC (mg/liter)[a]					
		pH 5			pH 7		
		Range	50%	90%	Range	50%	90%
B. melitensis (21)	Ciprofloxacin	≥8/≥8	≥8/≥8	≥8/≥8	1–2/2–≥8	1/≥8	2/≥8
	Ofloxacin	≥8/≥8	≥8/≥8	≥8/≥8	2–4/≥8	2/≥8	2/≥8
	Lomefloxacin	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8
	Temafloxacin	2–≥8/≥8	4/≥8	≥8/≥8	0.5–1/2–≥8	1/≥8	1/≥8
	Fleroxacin	≥8/≥8	≥8/≥8	≥8/≥8	1–4/≥8	2/≥8	2/≥8
	Sparfloxacin	2–≥8/≥8	4/≥8	≥8/≥8	0.5–1/2–≥8	0.5/≥8	1/≥8
B. abortus (4)	Ciprofloxacin	≥8/≥8	≥8/≥8	≥8/≥8	2–≥8/≥8	≥8/≥8	≥8/≥8
	Ofloxacin	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8
	Lomefloxacin	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8
	Temafloxacin	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8
	Fleroxacin	≥8/≥8	≥8/≥8	≥8/≥8	2–≥8/≥8	≥8/≥8	≥8/≥8
	Sparfloxacin	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8	≥8/≥8

[a] MICs are those obtained by the broth macrodilution method (10). The inoculum was 10^4 CFU/ml for all determinations. 50% and 90%, MIC and MBC for 50 and 90% of isolates, respectively.

and sparfloxacin MICs were 2 mg/liter. The MBCs indicate that for most strains, each compound had no bactericidal activity (MBCs for 90% of strains tested, ≥8 mg/liter at pHs 5 and 7). MICs for *B. abortus* were higher than those for *B. melitensis*. At pHs 5 and 7, all quinolones were inactive against the strains of *B. abortus* tested (MIC_{90}s, ≥8 mg/liter). No quinolone was bactericidal (MBCs, ≥8 mg/liter).

The ciprofloxacin MICs for *B. melitensis* found in this study were similar to those described in previous reports from Spain (3, 10), but they were higher than those in other reports from other countries performed by the same (13) and different (12) methodologies. Despite previously published data (13), ciprofloxacin was more active than ofloxacin against *B. melitensis*. Our results are in agreement with those of other recent studies (12). Even though fluoroquinolones show bactericidal activity in some strains (7, 9), results of this study indicate that fluorinated quinolones lack effective bactericidal activity against most strains of *Brucella* spp., and mainly against *B. abortus*, in spite of the fact that some strains are easily killed. In a previous study (7) performed at pH 7 and a final inoculum of 1.5×10^6 CFU/ml against susceptible strains of *B. melitensis*, ciprofloxacin proved to be markedly bactericidal, and at the MBCs, the time-kill curves indicated a very rapid bactericidal activity. This is in agreement with clinical therapeutic data. Ciprofloxacin has been clinically efficacious against some complications resulting from brucellosis (11), but therapeutic failures caused by the development of resistance to ciprofloxacin by *B. melitensis* have also been reported (1, 2, 12). In summary, fluoroquinolones must not be considered for use as a primary regimen for the treatment of brucellosis because of the selection of resistant strains and the lack of effective bactericidal activity at attainable intracellular concentrations, mainly at pH 5, the pH of phagolysosomes (16).

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