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Effect of Calcium and Magnesium Ions on the Susceptibility of *Pseudomonas* Species to Tetracycline, Gentamicin Polymyxin B, and Carbenicillin

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The effect of calcium and magnesium on the susceptibility of 13 species of *Pseudomonas* to tetracycline, gentamicin, polymyxin B, and carbenicillin was measured. The majority of the minimum inhibitory concentrations (MICs) of these antibiotics was increased if these cations were added to the test media. The increases in MICs caused by calcium or magnesium were similar, but the combination of both ions generally caused a greater change than either alone. Although the MIC of polymyxin B was most affected by calcium and magnesium, its interpretive susceptibilities (i.e., whether susceptible or resistant) were least changed. Susceptibility tests on *Pseudomonas* species probably should be done with Muller-Hinton broth supplemented with physiological concentrations of calcium and magnesium to better approximate the in vivo activity of these antibiotics. When the susceptibility tests were performed with Mueller-Hinton agar, the MICs were slightly less than those obtained with Mueller-Hinton broth supplemented with both cations but greater than those obtained with Mueller-Hinton broth supplemented with individual cations.

Susceptibility testing of *Pseudomonas aeruginosa* with the aminoglycosides polymyxin B and colistin is influenced by the presence of calcium and magnesium ions (2-9) The divalent cations affect the activity of these antibiotics, possibly through interaction with the cell wall of *P. aeruginosa* (1, 8, 11).

The effect of calcium and magnesium on the susceptibility of other species of *Pseudomonas* has not, to our knowledge, been investigated. These studies were designed to determine the influence of calcium and magnesium ions, individually and in combination, on the activity of tetracycline, gentamicin, polymyxin B, and carbenicillin, not only against *P. aeruginosa*, but also against 12 other species of *Pseudomonas*.

MATERIALS AND METHODS

The strains of *Pseudomonas* tested (shown in Table 1) were clinical isolates sent to the Center for Disease Control for identification. *P. aeruginosa* (ATCC 27853), recently adopted as a control strain for susceptibility testing, was included.

The antibiotics tested were tetracycline (Bristol Laboratories), gentamicin (Schering Corp.), and polymyxin B and carbenicillin (both from Pfizer, Inc.). All antibiotics were dissolved and diluted in Mueller-Hinton (M-H) broth (BBL), with additional cation(s) added according to the individual experiment.

The basic medium used for these studies was M-H broth (BBL). The concentrations of magnesium and calcium in this broth, as determined by atomic absorption spectrophotometry (Perkin-Elmer model 403 spectrophotometer), were 1.6×10^{-4} M and 8.4×10^{-5} M, respectively. For the comparative studies, the broth was supplemented with 2.10×10^{-3} mol of calcium (as $CaCl_2 \cdot 2H_2O$) and/or 1.40×10^{-3} mol of magnesium (as $MgCl_2 \cdot 6H_2O$), or 1.7% granulated agar (BBL). Therefore, the final concentration of calcium in the supplemented broth was 2.18×10^{-3} M (87 mg/liter), and the final concentration of magnesium was 1.56×10^{-3} M (37 mg/liter).

The broth dilution antibiotic susceptibility tests were performed in microtiter trays. The antibiotics were diluted serially in twofold-dilution steps in 0.05-ml volumes of M-H broth with and without additional calcium and magnesium as the diluent. Tetracycline, gentamicin, and polymyxin B were tested in final concentrations ranging from 128 to 0.015 μg/ml, and carbenicillin was tested in final concentrations of 1,024 to 0.12 μg/ml. Each microtiter plate was set up to test an antibiotic in: (i) M-H broth without additional cations, (ii) M-H broth plus calcium, (iii) M-H broth plus magnesium, (iv) M-H broth plus calcium and magnesium, and (v) a growth control containing only M-H broth plus inoculum. The inoculum, containing 10^5 colony-forming units/ml, was prepared in M-H broth containing the appropriate cation(s), and 0.05 ml was added to each well of the microtiter plate, making the final volume in each well 0.1 ml. All plates were incubated overnight at 35 C, except those containing *P. fluorescens*, which were incubated at room temperature. The minimum inhibitory concentration (MIC) was accepted as the

lowest concentration of antibiotic that inhibited visible growth.

Fifty-eight strains of *P. aeruginosa* were also tested with gentamicin by using an agar dilution method. The broth was supplemented with 1.7% agar, and gentamicin was added in final concentrations ranging in twofold dilutions from 128 to 0.015 μg/ml. The inocula (10^7 colony-forming units/ml) were added to the surface of the agar plates with a Steers replicating device (10), resulting in a final concentration of approximately 10^4 colony-forming units of each strain on each plate.

RESULTS

The effects of calcium, magnesium, and agar on the MICs of gentamicin for 58 strains of *P. aeruginosa* are shown in Fig. 1. In comparison to unsupplemented M-H broth, calcium, magnesium, agar, and a combination of calcium and magnesium, in that order, caused increases in the MICs of gentamicin. Although the differences between the effects of either calcium or magnesium were small, a combination of the two cations caused a much larger increase in the MICs than either alone. The effect of agar was less than the combination of calcium and magnesium, but greater than either cation alone. Whereas 98.3% of the strains tested in unsupplemented M-H were inhibited by 1 μg of gentamicin per ml, only 50, 22.4, 1.7, and 3.4% were inhibited when calcium, magnesium, a combination of the two cations, or agar, respectively, was added.

The effects of calcium and magnesium, singly or in combination, on MICs of tetracycline, gentamicin, polymyxin B, and carbenicillin for different species of *Pseudomonas* are shown in Tables 1 through 3. These data show that the MICs of two or more of the antibiotics were affected by these cations for most of the *Pseudomonas* species. The greatest number of changes was observed with tetracycline, but the largest changes were obtained with polymyxin B. In no case were the MICs decreased after the addition of cations.

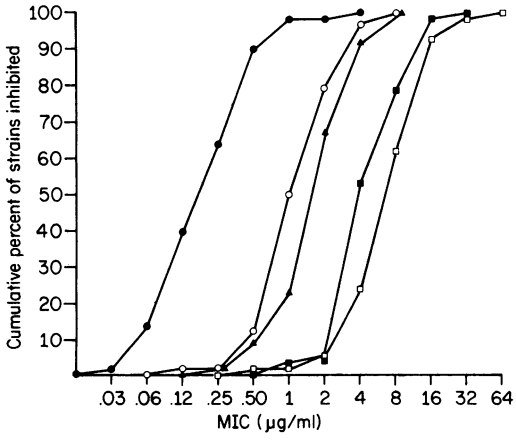

FIG. 1. *Effect of calcium and magnesium ions and agar on the MICs of gentamicin for 58 strains of pseudomonas aeruginosa. Symbols: ●, M-H broth; ○, M-H + Ca^{2+}; ▲, M-H + Mg^{2+}; □, M-H + Ca^{2+} + Mg^{2+}; ■, M-H + agar.*

TABLE 1. *Observed relative increase in MIC of antibiotics associated with the addition of calcium ions*

Species	Increase in MIC[a] (fold)			
	Tetracycline	Gentamicin	Polymyxin B	Carbenicillin
P. aeruginosa ATCC 27853	4	16	64	0
P. aeruginosa	8	32	8	4
P. fluorescens	8	0	2	0
P. putida	2	0	8	4
P. pseudomallei	4	2	0	0
P. cepacia	4	0	0	2
P. stutzeri	4	8	8	4
P. putrefaciens	8	2	8	8
P. maltophilia	4	8	128	4
P. diminuta	4	2	8	0
P. testosteroni	4	2	2	2
P. alcaligenes	4	0	0	8
P. pseudoalcaligenes	4	8	8	2
P. acidovorans	4	2	4	2

[a] Increase in MIC as compared to M-H broth without added cations.

TABLE 2. *Observed relative increase in MIC of antibiotics associated with the addition of magnesium ions*

Species	Increase in MIC[a] (fold)			
	Tetracycline	Gentamicin	Polymyxin B	Carbenicillin
P. aeruginosa ATCC 28753	4	8	32	0
P. aeruginosa	8	16	0	2
P. fluorescens	2	0	0	0
P. putida	2	0	4	4
P. pseudomallei	4	0	0	0
P. cepacia	4	0	0	0
P. stutzeri	2	8	0	2
P. putrefaciens	4	4	4	8
P. maltophilia	4	4	16	2
P. diminuta	0	0	4	0
P. testosteroni	4	2	2	2
P. alcaligenes	4	2	0	0
P. pseudoalcaligenes	4	4	8	2
P. acidovorans	8	2	2	2

[a] Increase in MIC as compared to M-H broth without added cations.

TABLE 3. *Observed relative increase in MIC of antibiotics associated with the addition of both calcium and magnesium ions*

Species	Increase in MIC[a] (fold)			
	Tetracycline	Gentamicin	Polymyxin B	Carbenicillin
P. aeruginosa ATCC 27853	8	32	256	2
P. aeruginosa	32	128	32	2
P. fluorescens	16	0	64	0
P. putida	4	0	32	4
P. pseudomallei	8	2	0	0
P. cepacia	8	0	0	2
P. stutzeri	16	64	32	4
P. putrefaciens	16	64	16	16
P. maltophilia	8	16	64	4
P. diminuta	≥8	2	16	0
P. testosteroni	8	4	4	4
P. alcaligenes	4	4	0	4
P. pseudoalcaligenes	4	16	8	2
P. acidovorans	16	4	8	4

[a] Increase in MIC as compared to M-H broth without added cations.

The effect that additional calcium and/or magnesium would have on the interpretive susceptibilities of the species tested, that is, whether they were susceptible, intermediate, or resistant, is shown in Tables 4 through 7. In terms of change from one interpretation to another, tetracycline (Table 4) was influenced the most, with the other three antibiotics being influenced about the same. If only major category changes are considered, that is, from susceptible to resistant, the susceptibility to tetracycline was again most affected, with two such changes upon the addition of either calcium or magnesium and five such changes with the addition of the combination of both cations. For gentamicin there were two changes from susceptible to resistant when both cations were added, and for carbenicillin there was one such change when calcium was added. With polymyxin B, no changes from susceptible to resistant were obtained.

DISCUSSION

These results demonstrate that calcium and magnesium ions, when added in normal human physiological concentrations to M-H broth, decrease the activity of tetracycline, gentamicin, polymyxin B, and, to a lesser extent, carbenicillin, not only against *P. aeruginosa*, but also against other *Pseudomonas* species. Because these organisms are playing an increasingly important role in nonsocomial infections, the effect of these cations should be considered when susceptibility tests are performed on any *Pseudomonas* species.

In the study of the 58 strains of *P. aeruginosa*, physiological concentrations of magnesium had a slightly greater effect on the MICs of gentamicin than did physiological concentrations of calcium, whereas in the study of the other species the reverse was true. In general, the effect of either cation alone is probably about the same. However, in both studies the effect of physiological concentrations of the combination of cations was much greater than either alone. The MICs of polymyxin B were affected most by the addition of these cations, and the MICs of carbenicillin were affected least; this is in agreement with the results reported by Davis and Iannetta (2, 3) but not with the results of Garrod and Waterworth (6), who reported that high concentrations of magnesium increased the resistance of *P. aeruginosa* to gentamicin and, to a lesser extent, to other aminoglycosides and polymyxin. Even though MICs of polymyxin B

TABLE 4. *Effect of cations on interpretive susceptibilities of Pseudomonas sp. to tetracycline*

Species	Tetracycline			
	Control[a]	Calcium[b]	Magnesium[c]	Calcium and magnesium[d]
P. aeruginosa ATCC 28753	I[e]	R	R	R
P. aeruginosa	S	I	I	R
P. fluorescens	S	S	S	S
P. putida	S	S	S	S
P. pseudomallei	S	I	I	R
P. cepacia	S	I	I	R
P. stutzeri	S	S	S	S
P. putrefaciens	S	S	S	S
P. maltophilia	S	R	R	R
P. diminuta	R	R	R	R
P. testosteroni	S	S	S	S
P. alcaligenes	S	R	R	R
P. pseudoalcaligenes	S	S	S	S
P. acidovorans	S	S	S	S
Total changes		6	6	6

[a] Control, M-H broth.

[b] Calcium, M-H + 2.1×10^{-3} mol of calcium as $CaCl_2 \cdot 2H_2O$.

[c] Magnesium, M-H + 1.4×10^{-3} mol of magnesium as $MgCl_2 \cdot 6H_2O$.

[d] Calcium plus magnesium, M-H + both cations in concentrations shown in *b* and *c*.

[e] S, Susceptible (MIC ≤ 4 μg/ml); I, intermediate (MIC 8 μg/ml); R, resistant (MIC ≥ 16 μg/ml).

TABLE 5. *Effect of cations on interpretative susceptibilities of Pseudomonas sp. to gentamicin*

Species	Gentamicin			
	Control[a]	Calcium[b]	Magnesium[c]	Calcium and magnesium[d]
P. aeruginosa ATCC 28753	S[e]	S	S	I
P. aeruginosa	S	S	S	S
P. fluorescens	S	S	S	S
P. putida	S	S	S	S
P. pseudomallei	R	R	R	R
P. cepacia	R	R	R	R
P. stutzeri	S	S	S	S
P. putrefaciens	S	S	S	S
P. maltophilia	S	I	S	R
P. diminuta	R	R	R	R
P. testosteroni	S	I	I	R
P. alcaligenes	R	R	R	R
P. pseudoalcaligenes	S	S	S	S
P. acidovorans	I	R	R	R
Total changes		3	2	4

[a] Control, M-H broth.

[b] Calcium, M-H + 2.1×10^{-3} mol of calcium as $CaCl_2 \cdot 2H_2O$.

[c] Magnesium, M-H + 1.4×10^{-3} mol of magnesium as $MgCl_2 \cdot 6H_2O$.

[d] Calcium plus magnesium, M-H + both cations in concentrations shown in *b* and *c*.

[e] S, Susceptible (MIC $\leq$ 4 μg/ml); I, intermediate (MIC 8 μg/ml); R, resistant (MIC $\geq$ 16 μg/ml).

TABLE 6. *Effect of cations on interpretive susceptibilities of Pseudomonas sp. to polymyxin B*

Species	Polymyxin B			
	Control[a]	Calcium[b]	Magnesium[c]	Calcium and magnesium[d]
P. aeruginosa ATCC 28753	S[e]	S	S	S
P. aeruginosa	S	S	S	S
P. fluorescens	S	S	S	S
P. putida	S	S	S	S
P. pseudomallei	R	R	R	R
P. cepacia	R	R	R	R
P. stutzeri	S	S	S	S
P. putrefaciens	I	R	R	R
P. maltophilia	S	S	S	S
P. diminuta	I	R	R	R
P. testosteroni	I	R	R	R
P. alcaligenes	S	S	S	S
P. pseudoalcaligenes	S	S	S	S
P. acidovorans	S	S	S	S
Total changes		3	3	3

[a] Control, M-H broth.

[b] Calcium, M-H + 2.1×10^{-3} mol of calcium as $CaCl_2 \cdot 2H_2O$.

[c] Magnesium, M-H + 1.4×10^{-3} mol of magnesium as $MgCl_2 \cdot 6H_2O$.

[d] Calcium plus magnesium, M-H + both cations in concentrations shown in *b* and *c*.

[e] S, Susceptible (MIC $\leq$ 4 μg/ml); I, intermediate (MIC 8 μg/ml); R, resistant (MIC $\geq$ 16 μg/ml).

TABLE 7. *Effect of cations on interpretive susceptibilities of Pseudomonas sp. to carbenicillin*

Species	Carbenicillin			
	Control[a]	Calcium[b]	Magnesium[c]	Calcium and magnesium[d]
P. aeruginosa ATCC 28753	S[e]	S	S	S
P. aeruginosa	S	S	S	S
P. fluorescens	R	R	R	R
P. putida	S	I	I	I
P. pseudomallei	I	I	I	I
P. cepacia	S	S	S	S
P. stutzeri	S	S	S	S
P. putrefaciens	S	S	S	S
P. maltophilia	I	R	R	R
P. diminuta	S	S	S	S
P. testosteroni	S	S	S	S
P. alcaligenes	S	R	S	I
P. pseudoalcaligenes	S	S	S	S
P. acidovorans	S	S	S	I
Total changes		3	2	4

[a] Control, M-H broth.

[b] Calcium, M-H + 2.1×10^{-3} mol of calcium as $CaCl_2 \cdot 2H_2O$.

[c] Magnesium, M-H + 1.4×10^{-3} mol of magnesium as $MgCl_2 \cdot 6H_2O$.

[d] Calcium plus magnesium, M-H + both cations in concentrations shown in *b* and *c*.

[e] S, Susceptible (MIC $\leq$ 64 μg/ml); I, intermediate (MIC 128 μg/ml); R, resistant (MIC $\geq$ 256 μg/ml).

were influenced the most by these cations, the clinical interprepations of the MICs were the least affected; the susceptibility of three strains changed from intermediate to resistant. It is possible that the effect of individual cations may vary considerably from strain to strain.

The lot of M-H broth used for these tests contained calcium and magnesium ions in concentrations much lower than in normal human serum. By adding the cations in the concentrations used in this study, we approximated the physiological concentrations of these ions. Possibly, MICs obtained by adding both cations more accurately indicated the in vivo effectiveness of the antibiotic tested. If this is true, susceptibility tests with these antibiotics on *Pseudomonas* species should be done in M-H broth supplemented with calcium and magnesium, or by agar dilution, in which results approximate those obtained in M-H broth supplemented with physiological concentrations of calcium and magnesium.

These results could have other clinical implications. MICs of gentamicin for the 58 strains of *P. aeruginosa* tested in this study were 4 μg/ml or less when tested in unsupplemented M-H broth; these levels are readily achievable in the blood. However, if the tests were performed in M-H broth supplemented with physiological concentrations of both calcium and magnesium, 37.9% of the strains had MICs of 16 or 32 μg/ml, levels which would be toxic to a patient. Clinical studies should be performed to determine if the higher MICs obtained in the presence of physiological concentrations of both calcium and magnesium correlate more closely with patient response.

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