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Effect of Divalent Cation Concentrations on the Antibiotic Susceptibilities of Nonfermenters Other than *Pseudomonas aeruginosa*

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The effects of supplementing Mueller-Hinton broth with calcium and magnesium on the minimal inhibitory concentrations (MICs) of eight aminoglycosides, colistin, tetracycline, and carbenicillin for 11 nonfermenters other than *Pseudomonas aeruginosa* were studied and compared with the effects for *Escherichia coli* and *P. aeruginosa*. MICs were simultaneously performed in unsupplemented Mueller-Hinton broth and Mueller-Hinton broth supplemented to contain 5 mg of calcium and 2.5 mg of magnesium per dl. Changes in MICs were expressed as the increases in the number of $\log_2$ concentrations caused by supplementation. The usual increases in MICs of aminoglycosides caused by supplementation were: zero concentrations for *E. coli*, one to six concentrations for *P. aeruginosa*, and one to two concentrations for most other nonfermenters. The largest increases (five to six concentrations) were observed with gentamicin and *P. aeruginosa*. The usual increases in MICs of colistin were: zero concentrations for *E. coli*, two concentrations for *P. aeruginosa*, and one to two concentrations for other nonfermenters. Increases in MICs of tetracycline were: one to five concentrations for all organisms tested. The usual increases in MICs of carbenicillin were: zero concentrations for *E. coli* and *P. aeruginosa* and zero to two concentrations for other nonfermenters. These observations indicated that supplementation of Mueller-Hinton broth to contain recommended concentrations of calcium and magnesium had little effect on MICs of aminoglycosides and colistin for *E. coli* but increased MICs for most nonfermenters, increased MICs of tetracycline for *E. coli* and all nonfermenters, and had little effect on MICs of carbenicillin for *E. coli* and *P. aeruginosa* but increased the MICs for several nonfermenters other than *P. aeruginosa*.

The antagonistic effect of divalent cations on the antibacterial activity of aminoglycosides (7), polymyxins (12), and tetracycline (18) has been recognized for many years. The largest number of reports (1, 3, 5, 8, 9, 11, 15, 19) have documented the marked effects of the calcium and magnesium content in broth media on the activity of gentamicin against *Pseudomonas aeruginosa* but not *Enterobacteriaceae*. Because commercially available media vary considerably in their calcium and magnesium contents (8, 9, 11, 15, 17), it has been recommended that susceptibility testing by broth dilution techniques utilize Mueller-Hinton broth (MHB), a medium low in calcium and magnesium content, which is supplemented to contain physiological concentrations of those cations (3, 9, 15, 16).

This study was designed to determine the effects of supplementing MHB with calcium and magnesium on the in vitro activities of eight aminoglycoside antibiotics, colistin, and tetracycline against nonfermenters other than *P. aeruginosa*; strains of *Escherichia coli* and *P. aeruginosa* were included for comparison. Carbenicillin, an antibiotic active against *P. aeruginosa*, but not markedly affected by calcium and magnesium (3, 5, 15, 19), was simultaneously studied.

MATERIALS AND METHODS

Culture medium. A single lot of MHB (Difco Laboratories, Detroit, Mich.) was used for all susceptibility tests. It contained approximately 1.0 mg of calcium and 0.5 mg of magnesum per dl. For each series of tests, half of the medium prepared was supplemented with $CaCl_2$ and $MgCl_2$ to contain 5.0 mg of calcium and 2.5 mg of magnesium per dl, and half remained unsupplemented.

Organisms. Five strains each of *E. coli*, *P. aeruginosa*, *Pseudomonas maltophilia*, *Pseudomonas cepacia*, *Pseudomonas fluorescens-putida*, *Pseudomo-*

nas stutzeri, Acinetobacter calcoaceticus var. *anitratus, Acinetobacter calcoaceticus* var. *lwoffi, Alcaligenes odorans, Achromobacter xylosoxidans, Bordetella bronchiseptica, Moraxella* sp. and *Flavobacterium meningosepticum* were studied.

Antibiotics. The antibiotics tested were streptomycin, kanamycin, gentamicin, tobramycin, amikacin, sisomicin, netilmicin, Sch 21420, colistin, tetracycline, and carbenicillin. Laboratory standards of streptomycin and tobramycin were obtained from Eli Lilly & Co. (Indianapolis, Ind.). Kanamycin and amikacin were obtained from Bristol Laboratories (Syracuse, N.Y.). Gentamicin, sisomicin, netilmicin, and Sch 21420 were obtained from Schering Corp. (Kenilworth, N.J.). Colistin was obtained from Warner/Chilcott Laboratories (Morris Plains, N.J.). Tetracycline was obtained from Lederle Labortories (Pearl River, N.Y.). Carbenicillin was obtained from Beecham Products (Pittsburgh, Pa.). Laboratory standards were dissolved and diluted to concentrations of 1,000 μg/ml (10,000 μg of carbenicillin per ml) in sterile distilled water, sterilized by passage through 0.2-μm filters (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) and stored at −20°C until used.

Broth dilution susceptibility tests. Minimal inhibitory concentrations (MICs) of all eleven antibiotics were determined simultaneously in both supplemented and unsupplemented MHB for each organism using the Dynatech MIC-2000 (Dynatech Laboratories, Inc., Alexandria, Va.) microdilution method (10). Organisms were tested in groups of five to ten. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as control strains.

Antibiotic-containing microdilution plates were prepared in advance, checked for sterility, and then stored at −70°C until used. Antibiotic concentrations were 32 to 0.03 μg/ml for all antibiotics other than carbenicillin; carbenicillin concentrations were 512 to 0.5 μg/ml. The inoculum was approximately 5×10^5 colony-forming units per ml. Wells without antibiotic served as growth controls. Inoculated microdilution plates were incubated overnight in a room-air incubator at 37°C. The MICs were defined as the lowest concentrations of antibiotic that inhibited visible growth.

RESULTS

The changes in MICs were expressed as the increases in the number of $\log_2$ concentrations caused by supplementing MHB and are shown in Tables 1 and 2.

MICs of aminoglycosides for *E. coli* were the same in both media for 27 of 40 determinations; 10 were higher and 3 were lower in supplemented than in unsupplemented MHB. For *P. aeruginosa*, all MICs were higher in supplemented MHB; the effect was greatest with gentamicin (five to six concentrations) and least with streptomycin (one concentration). For nonfermenters other than *P. aeruginosa*, supplementation usually raised MICs by one to two concentrations. Exceptions were that MICs for *A. calcoaceticus* var. *anitratus* were generally unaffected by supplementation and that MICs of tobramycin for *A. odorans* were four or more concentrations higher in supplemented MHB. The effect on MICs for *P. cepacia, A. xylosoxidans*, and *F. meningosepticum* could not be determined because all strains were resistant to 32 μg of the antibiotics tested per ml.

MICs of colistin for *E. coli* were zero to one concentration higher in supplemented MHB. For *P. aeruginosa* and other nonfermenters, the usual increase was about two concentrations. The effect on MICs for *P. cepacia* and *F. meningosepticum* could not be determined because all strains were resistant to 32 μg of colistin per ml.

MICs of tetracycline were higher in supplemented MHB for every strain tested.

MICs of carbenicillin in supplemented and in unsupplemented MHB were similar for all organisms tested except that supplementation resulted in higher MICs for *P. maltophilia, A. calcoaceticus* var. *lwoffi*, and *B. bronchiseptica*.

DISCUSSION

Since gentamicin has become available, studies on its in vitro activity against *Enterobacteriaceae* and *P. aeruginosa* have indicated variations in MICs depending upon which medium was used and whether or not agar was added to a given medium. The variations were attributed primarily to differences in cation content (1, 3, 5, 8, 9, 11, 15, 17, 19). There was a nonspecific ionic strength-dependent effect in which higher salt concentrations resulted in higher MICs for all organisms tested. There also was a divalent cation concentration-dependent effect which was out of proportion to changes in ionic strength and which was specific for *P. aeruginosa*; slight increases in calcium and magnesium contents caused marked increases in MICs (1, 8, 9, 11). Supplementation of MHB to physiological concentrations of calcium and magnesium or the addition of cation-containing agar yielded MICs of gentamicin which were approximately 32-fold higher than those observed in unsupplemented MHB (3, 8, 9, 11, 15). The calcium and magnesium probably reduced the binding of gentamicin to *P. aeruginosa* (14) or stabilized the cell wall to the action of gentamicin (9, 19). Although other aminoglycosides were not studied as thoroughly as gentamicin, there were data to indicate that streptomycin (11), dihydrostreptomycin (1), kanamycin (11), tobramycin (4, 6, 15, 17, 19), amikacin (15, 17), and sisomicin (19) were similarly antagonized by calcium and magnesium, but probably to a lesser degree, in their activities against *P. aeruginosa*. One study (3) examined the effect of supplementing MHB on the MICs of gentamicin, but not other aminogly-

TABLE 1. *Changes in MICs expressed as the increases in number of log_2 concentrations caused by supplementing MHB with calcium and magnesium: E. coli and Pseudomonas species*

Antibiotic	*E. coli*	*P. aeruginosa*	*P. maltophilia*	*P. cepacia*	*P. fluorescens-putida*	*P. stutzeri*
Streptomycin	1[a] (0–1)	1 (1)	R[b] (1)	R	1 (0–1)	1 (0–2)
Kanamycin	0 (0)	R (≥1–3)	R	R	1 (0–1)	1 (0–1)
Gentamicin	0 (−1–0)	5 (5–6)	2 (1–3)	R	1 (0–4)	1 (0–2)
Tobramycin	0 (0–1)	2 (2–6)	1 (0–2)	R	1 (0–2)	1 (0–1)
Amikacin	0 (0–1)	2 (1–2)	1 (1–2)	R	1 (0–2)	1 (0–1)
Sisomicin	0 (−1–0)	4 (3–4)	1 (1–2)	R	1 (0–2)	0 (0–1)
Netilmicin	0 (−1–1)	4 (4–5)	1 (0–1)	R	1 (0–4)	1 (0–1)
Sch 21420	1 (0–1)	3 (2–3)	1 (1–2)	R (≥1)	1 (0–1)	1 (0–2)
Colistin	0 (0–1)	2 (2)	1 (0–3)	R	2 (2–3)	1 (1–2)
Tetracycline	1 (1–2)	R (≥3–5)	3 (3)	R (≥3–4)	3 (2–4)	2 (1–4)
Carbenicillin	0 (−1–0)	0 (0–1)	3 (2–4)	R (0)	R (0)	1 (−1–2)

[a] Number of concentrations: median (range in parentheses). At least three of five strains had MICs within the range of concentrations tested in both supplemented and unsupplemented MHB.

[b] R, Three or more strains were resistant to 32 μg (512 μg of carbencillin) per ml in supplemented MHB. The range in parentheses is for those strains which had MICs within the range studied in unsupplemented MHB.

cosides, against single strains of various *Pseudomonas* species other than *P. aeruginosa*; increases in MICs caused by supplementation were variable and usually smaller than those observed with *P. aeruginosa*. In the present study, supplementation of MHB to currently recommended (16) concentrations of calcium and magnesium increased MICs of gentamicin against *P. aeruginosa* by approximately 32-fold but had minimal effects on the MICs for *E. coli*. MICs of other aminoglycosides for *P. aeruginosa* were affected similarly but to a lesser extent. The effect on MICs of the aminoglycosides against nonfermenters other than *P. aeruginosa* varied by organism but was similar for most strains of each species. Among the *Pseudomonas* species, *P. stutzeri* was least affected. Among all the nonfermenters, only MICs for *A. calcoaceticus* var. *anitratus* were virtually unaffected. In general, MICs of gentamicin were increased more by supplementation than those of the other aminoglycosides, although tobramycin was antagonized more than gentamicin in activity against *A. odorans*.

The antagonistic effect of divalent cations on the antibacterial action of polymyxins against *P. aeruginosa* has also been well recognized (2, 3, 12, 13, 15). Like the aminoglycosides, the effect seemed to be on the uptake of drug or on the interaction of the cations with the cell wall (2, 12, 13). In one report, the antagonistic effect was less than that observed with gentamicin (8), whereas in another it was greater (5), and in a third variable (3). The effect of supplementing MHB on the MICs of polymyxin B for single strains of various *Pseudomonas* species other than *P. aeruginosa* was variable as it was for gentamcin (3). In the present study, supplementation had little or no effect on MICs for *E. coli*. The MICs for all strains of *P. aeruginosa* were increased fourfold. The effects on MICs for other nonfermenters were similar (zero- to eightfold) in magnitude.

Divalent cations have also been observed to inhibit the antibacterial activity of tetracyclines against *P. aeruginosa* (3, 15). Unlike the aminoglycosides and polymyxins, however, this effect was not unique to *P. aeruginosa* and was presumably related to combining of the drug with those ions which were present in the media (18). In the present study, as in another (3), supplementation of MHB resulted in increased MICs for every strain of all organisms tested, including *E. coli*, from 2- to 32-fold.

The antibacterial activity of carbenicillin against *Enterobacteriaceae* and *P. aeruginosa*

TABLE 2. *Changes in MICs expressed as the increases in number of log_2 concentrations caused by supplementing MHB with calcium and magnesium: nonfermenters other than Pseudomonas species*

Antibiotic	*A. anitratus*	*A. lwoffi*	*A. odorans*	*A. xylosoxidans*	*B. bronchiseptica*	*Moraxella* sp.	*F. miningosepticum*
Streptomycin	1[a] (−1-1)	1 (0-2)	R[b] (≥1-2)	R	R (≥2)	1 (0-1)	R
Kanamycin	1 (0-1)	1 (1-2)	R (≥1-2)	R	1 (1-2)	1 (0-3)	R
Gentamicin	0 (−1-1)	2 (2)	2 (1-2)	R	2 (1-2)	S[c] (0-≥2)	R
Tobramycin	0 (0-1)	2 (2)	R (≥4-6)	R	1 (1-2)	S (≥1)	R
Amikacin	0 (0-1)	1 (1)	0 (0-1)	R	1 (1)	2 (0-3)	R
Sisomicin	0 (0)	1 (1-3)	2 (0-2)	R	2 (1-2)	S (≥1)	R
Netilmicin	1 (−1-1)	0 (0-2)	1 (1)	R	1 (1)	S (0-≥2)	R
Sch 21420	1 (0-2)	1 (1-2)	1 (0-2)	R	2 (1-2)	2 (1-3)	R (≥1)
Colistin	1 (0-2)	2 (1-2)	2 (1-3)	2 (2)	2 (0-3)	1 (0-2)	R
Tetracycline	2 (1-2)	3 (2-3)	4 (4-5)	R (≥1)	3 (2-3)	2 (1-3)	R (≥1)
Carbenicillin	0 (−1-0)	1 (0-3)	0 (0-2)	0 (0-2)	1 (1-2)	S (0)	0 (0)

[a] Number of concentrations: median (range in parentheses). At least three of five strains had MICs within the range of concentrations tested in both supplemented and unsupplemented MHB.

[b] R, Three or more strains were resistant to 32 μg (512 μg of carbenicillin) per ml in supplemented MHB. The range in parentheses is for those strains which had MICs within the range studied in unsupplemented MHB.

[c] S, Three or more strains were inhibited by 0.03 μg (0.5 μg of carbenicillin) or less per ml in unsupplemented MHB. The range in parentheses is for those strains which had MICs within the range studied in supplemented MHB.

has been generally considered to be unaffected by physiological concentrations of calcium and magnesium (3, 5, 15). In one study (3) of *Pseudomonas* species, however, MICs for some strains were markedly affected. In the present study, there was also no significant effect on the MICs for *E. coli* and *P. aeruginosa*, but the MICs for some strains of other nonfermenters, particularly *P. maltophilia*, were increased by supplementation.

We agree with others (3, 9, 15) that the calcium and magnesium contents of commercially available media used for susceptibility testing should be controlled. We also recognize that other factors present in media may also influence MICs (17) and that it would be appropriate to establish acceptable control limits for MICs utilizing a number of antibiotic-reference strain combinations which could be applied to any medium used for that purpose (8, 9, 15, 17).

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