ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1981, p. 987-992
0066-4804/81/060987-06$02.00/0

Vol. 19, No. 6

Effect of Ionized Calcium and Soluble Magnesium on the Predictability of the Performance of Mueller-Hinton Agar Susceptibility Testing of *Pseudomonas aeruginosa* with Gentamicin

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Received 11 February 1981/Accepted 31 March 1981

The soluble and ionized calcium and magnesium contents of 18 lots of Mueller-Hinton agar medium from three different manufacturers were analyzed, and the results were correlated with medium performance. A standardized disk diffusion test, with *Pseudomonas aeruginosa* (ATCC 27853) and a 10-μg gentamicin disk, served as an indicator of medium performance. Zone diameters correlated well with the ionized calcium values and the sum of the ionized calcium and soluble magnesium values in the different lots ($r = -0.88$ for both). Zone diameters correlated poorly with ionized magnesium values ($r = -0.57$), which were best described by a curvilinear relationship. Supplementation of lots of Mueller-Hinton agar medium with equivalent amounts of calcium and magnesium as the chloride, gluconate, or glycerophosphate salts produced identical decreases in zone sizes. Adjustment of deficient lots of Mueller-Hinton agar medium with ionized calcium or soluble magnesium or both (as the gluconate salts), to match the concentrations in lots that provided satisfactory zone sizes (17 to 19 mm), resulted in performance comparable to that of the control lots. Sixteen strains of *Pseudomonas aeruginosa*, ranging from resistant to susceptible, responded to cation adjustment in the same manner as the ATCC quality control strain. Satisfactory medium performance can obviously be assured by biological means in aminoglycoside susceptibility testing of *Pseudomonas aeruginosa* on Mueller-Hinton medium; however, cation adjustment of medium to predetermined levels of ionized calcium and soluble magnesium can now also provide desirable performance levels for *P. aeruginosa* on Mueller-Hinton medium.

Recently, Kenny et al. (2) described some of the quantitative and qualitative aspects of the relationship between the cation content of Mueller-Hinton medium and the susceptibility of *Pseudomonas aeruginosa* to aminoglycosides. An interdependence of calcium and magnesium was demonstrated, and it was found that the soluble rather than the total cations affected the system. More specifically, the concentration of ionized calcium, probably the biologically active fraction, correlated best with gentamicin susceptibility. It was hypothesized further that ionized magnesium would also relate in the same way, i.e., resistance to gentamicin would increase directly with the concentration of ionized magnesium in the medium.

This report further defines the role of these cations in the interaction between aminoglycosides, particularly gentamicin, and *P. aeruginosa* in in vitro susceptibility testing. Inhibition zone sizes were directly correlated with the concentrations of ionized calcium and soluble magnesium in Mueller-Hinton media. The relationships defined in this paper were used to manipulate Mueller-Hinton agar medium to a desired and predictable performance level.

MATERIALS AND METHODS

Media and bacteria. The soluble and ionized calcium and magnesium contents of 18 lots of Mueller-Hinton agar medium were analyzed, and their performance in testing the susceptibility of *P. aeruginosa* to gentamicin was evaluated. Media were prepared and separated into soluble and insoluble fractions as previously described (2). Difco lots (Difco Laboratories, Detroit, Mich.) were numbered 620275, 615800, 564372, 640657, 648618, 652161, and 655476. BBL lots (BBL Microbiology Systems, Cockeysville, Md.) were designated I1DIFZ, G1DIAO, D1DHOL, A1DHQZ,

E1DHQS, and L5DART. GIBCO lots (GIBCO Diagnostics, Madison, Wis.) were numbered 870480, 870304, 870442, 870402, and 970011.

P. aeruginosa ATCC strain 27853, the standard gentamicin-susceptible quality control strain (3), was used to evaluate medium performance in all experiments. Sixteen other strains of *P. aeruginosa* were used to determine whether the response of the standard strain (ATCC 27853) was representative of a population of *P. aeruginosa* isolates challenged with gentamicin. One of these strains, from the Mayo Clinic, had been previously studied for its response to agar supplementation with calcium and magnesium (4). The Ellsworth strain was originally obtained from Pfizer Therapeutics Inc., New York, N.Y., for use in other drug studies. Fourteen of the strains were clinical isolates from the culture collection of the Clinical Microbiology Laboratory, University Hospital, Seattle, Wash. *Escherichia coli* ATCC 25922 was also used for quality control in all experiments.

Susceptibility testing. The standardized disk diffusion test was performed as recommended (3). Each medium in all experiments was prepared in duplicate, with each sample individually weighed and prepared separately. For each study of gentamicin activity against *P. aeruginosa*, two antibiotic disks were placed on plates prepared from duplicate medium samples. The average of the zone diameters read from the four separate disks was reported.

Ionized calcium was measured with an ion-specific electrode (Orion Research Inc., Cambridge, Mass.), and the total soluble calcium and magnesium in the fluid phase of the medium was determined by atomic absorption spectroscopy as previously described (2). All determinations were made after autoclaving media.

Ionized magnesium was measured colorimetrically by the method of Scarpa (6), as modified in our laboratory (E. Casillas and M. A. Kenny, submitted for publication). Dual wavelength spectroscopy (with 550 and 600 nm, respectively, for test and blank) was performed with an ABA-100 analyzer (Abbott Laboratories, North Chicago, Ill.). The blank solutions were prepared by treating medium fluid with Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.) cation-exchange resin. The precision of analysis was 1.00 ± 0.06 meq/liter ($\bar{X} \pm$ standard deviation) based on 20 determinations made on 20 successive days.

All cation concentration data on media and medium fractions were expressed in milliequivalents per liter to represent the chemically active quantity. Factors for the conversion of milliequivalents to milligrams per liter are 20.04 for calcium and 12.16 for magnesium (1).

Media supplementation. The influence of the anionic components of calcium and magnesium salts on the availability of these cations in supplemented Mueller-Hinton agar media was investigated. In these experiments, Difco lot 655476 and BBL lot E1DHQS were supplemented by addition of stock solutions of calcium and magnesium as chloride, gluconate, or glycerophosphate salts. Approximate final concentrations of 1.05 meq of ionized calcium per liter and 1.71 meq of total soluble magnesium per liter were obtained in the media. These "target" concentrations were based on preliminary assessments of cation concentrations in Mueller-Hinton media that were performing within recommended standards for aminoglycoside susceptibility testing with fully susceptible *P. aeruginosa* ATCC 27853. We chose media that yielded gentamicin inhibition zone diameters of 17 to 19 mm as reference media. These limits are within the performance standards established for *P. aeruginosa* ATCC 27853 (5). Lots of medium in other experiments were supplemented only with calcium and magnesium gluconate. Calcium and magnesium supplements and concentrations achieved are given in the appropriate tables and figures. Zones of inhibition around gentamicin disks and cation contents in supplemented medium were compared with those of the same lot of unsupplemented control medium.

RESULTS

Correlation of cation composition of media with gentamicin zone diameters. The contents of 18 lots of Mueller-Hinton agar medium from three manufacturers were analyzed for total soluble calcium, soluble ionized calcium, total soluble magnesium, and soluble ionized magnesium. These results were compared with those obtained from the gentamicin susceptibility testing of *P. aeruginosa* done on these media (Table 1).

There was a considerable range of values for calcium and magnesium in the lots of Mueller-Hinton agar medium examined. Soluble calcium content ranged from 1.14 meq/liter to 3.14 meq/liter. Although the GIBCO lots tended to be somewhat lower in soluble calcium than the Difco and BBL lots, these differences were not apparent for ionized calcium content. The relationship of ionized calcium to soluble calcium separated the media into two distinct groups. In all of the GIBCO lots, the ionized calcium was approximately 61% (57 to 66%) of the soluble calcium, but the ionized calcium was approximately 35% (29 to 40%) of the soluble calcium in the BBL lots; both groups were represented among the Difco lots. In contrast, the ionized magnesium content ranged from 0.19 meq/liter to 2.79 meq/liter, or from 11 to 122% of the soluble magnesium. (The latter figure, for BBL lot E1DHQS, results from the variation in both medium preparation and assay.)

The inverse relationship of zone diameter to cation content of the 18 lots of media, as described by correlation coefficients (r), ranged from -0.42 to -0.88. The best correlations were found for ionized calcium and for the sum of ionized calcium plus soluble magnesium: -0.88 in both cases. The results with ionized magnesium were not as convincing. The correlation was best described by a curvilinear regression, but even so, the r value was low (-0.53).

Media supplementation. Unsupplemented

TABLE 1. *Comparison of soluble and ionized cation contents of Mueller-Hinton agar media with susceptibility of P. aeruginosa ATCC 27853 to gentamicin*[a]

Lot of medium	Calcium[b]		Magnesium[b]		Calcium plus magnesium		Ionized calcium plus soluble magnesium	Gentamicin 10-μg disk zone (mm)
	Soluble	Ionized	Soluble	Ionized	Soluble	Ionized		
Difco								
620275	2.83	1.70 (60)	2.30	1.35 (59)	5.13	3.05	4.00	15.4
652161	2.13	0.65 (31)	1.97	0.36 (18)	4.10	1.01	2.62	19.4
648618	2.03	1.15 (57)	1.75	0.23 (13)	3.78	1.38	2.90	18.0
615800	2.23	1.42 (64)	2.99	2.53 (85)	5.22	3.95	4.41	15.8
655476	1.75	0.65 (37)	1.42	0.53 (37)	3.17	1.18	2.07	19.9
564372	1.33	0.84 (63)	2.91	2.79 (96)	4.24	3.63	3.75	18.2
640657	2.08	1.19 (57)	1.72	1.24 (72)	3.80	2.43	2.91	17.2
BBL								
I1DIFZ	3.14	1.21 (39)	1.96	0.83 (42)	5.10	2.04	3.17	17.7
G1DIAO	2.87	1.15 (40)	1.83	0.86 (47)	4.70	2.01	2.98	17.8
D1DHOL	2.68	0.98 (37)	1.52	0.93 (61)	4.20	1.91	2.50	18.3
A1DHQZ	2.69	0.95 (35)	1.54	0.45 (29)	4.23	1.40	2.49	17.4
E1DHQS	1.78	0.53 (30)	0.51	0.62 (122)	2.29	1.15	1.04	22.0
L5DART	2.82	0.81 (29)	1.26	0.38 (30)	4.08	1.19	2.07	21.0
GIBCO								
870480	1.72	1.01 (59)	1.72	0.19 (11)	3.44	1.20	2.73	17.5
870304	1.55	0.88 (57)	1.55	[c]	3.10	[d]	2.43	18.2
870442	1.19	0.76 (64)	1.12	0.26 (23)	2.31	1.02	1.88	20.8
870402	1.14	0.75 (66)	1.07	0.54 (50)	2.21	1.28	1.82	20.8
970011	1.91	1.12 (59)	1.80	0.41 (23)	3.71	1.53	2.92	18.2
r[e]	−0.42	−0.88	−0.77	−0.53	−0.77	−0.75	−0.88	

[a] Data are given as milliequivalents per liter except as otherwise indicated.

[b] Soluble and ionized cations were in the fluid phase of prepared media. Parentheses indicate percent of soluble cation.

[c] Not assayed because of technical difficulty.

[d] Not reportable because of unassayable ionized magnesium value.

[e] Correlation of zone diameters with cation concentrations; correlation coefficients (*r*) were calculated by the least-squares method.

media that yielded zones of inhibition around gentamicin disks of 17 to 19 mm with *P. aeruginosa* ATCC 27853 were selected as control lots (Table 1). We arbitrarily considered these to be performing satisfactorily, even though our criterion was more stringent than that of the National Committee for Clinical Laboratory Standards (zones of 16 to 21 mm). For these nine lots, the average ionized calcium concentration was 1.05 ± 0.13 meq/liter, and the average soluble magnesium concentration was 1.71 ± 0.15 meq/liter.

(i) Effect of anions. Because the distribution of cations into the ionized fraction could have been affected by the anionic component of the calcium or magnesium salt, we analyzed media with these cations added as either chlorides, gluconates, or glycerophosphates (Table 2). Two lots of Mueller-Hinton agar medium were supplemented (Difco 655476 and BBL E1DHQS), and their performance was tested with three strains of *P. aeruginosa*, ATCC 27853, Mayo, and Ellsworth strains. After supplementation, the cation contents of both lots were similar, i.e., analyses of soluble and ionized calcium or magnesium agreed within experimental error, irrespective of the anionic component of the cation source. Additionally, cations added in the initial preparation of the Mueller-Hinton agar were completely recovered in all cases in the fluid phase, and the added anionic component did not upset the existing balance between the ionized and soluble cation fractions. No significant differences in gentamicin zone diameters were exhibited by the three strains of *P. aeruginosa* on medium supplemented with calcium and magnesium as either chlorides, gluconates, or glycerophosphates.

(ii) Addition of calcium and magnesium. Preliminary experiments indicated that performance of Mueller-Hinton agar medium could not be reliably predicted when either cation alone was added. These experiments did demonstrate, however, that a medium could be sup-

TABLE 2. *Effects of calcium and magnesium added to Mueller-Hinton agars as different anion salts on the susceptibility of P. aeruginosa to gentamicin*

Lot of medium	Supplemented anion	Cations (meq/liter)[a]				Gentamicin[b] zone diameters for *P. aeruginosa* strain:		
		Ca_s	Ca_i	Mg_s	Mg_i	ATCC 27853	Mayo	Ellsworth
Difco 655476	None	1.75	0.65	1.42	0.53	20.3	21.8	18.0
	Chloride	2.77	1.14	1.60	0.73	18.4	20.5	16.3
	Gluconate	2.67	1.10	1.60	0.67	18.7	20.7	16.7
	Glycerophosphate	2.67	1.09	1.60	0.75	18.8	20.4	16.3
BBL E1DHQS	None	1.78	0.53	0.51	0.62	22.5	23.1	20.7
	Chloride	3.52	1.25	1.78	1.74	18.9	20.2	17.3
	Gluconate	3.32	1.14	1.62	1.57	19.6	20.7	17.6
	Glycerophosphate	3.29	1.10	1.72	1.72	19.5	21.2	17.2

[a] Target concentrations for ionized (*i*) calcium and soluble (*s*) magnesium were 1.05 ± 0.13 meq/liter and 1.71 ± 0.15 meq/liter, respectively.

[b] Gentamicin disks contained 10 μg of antibiotic.

plemented to a predictable level of ionized calcium and soluble magnesium.

Six lots of media originally deficient in ionized calcium or soluble magnesium or both were selected as test lots for cation supplementation. Zones of inhibition with gentamicin disks were greater than 19 mm with *P. aeruginosa* ATCC 27853 on these media. Test media were supplemented with calcium and magnesium gluconate to match target concentrations of 1.05 meq of ionized calcium per liter and 1.71 meq of soluble magnesium per liter, the average cation concentrations of the control media. After cation additions, gentamicin zone diameters averaged 18.4 ± 0.6 mm (Fig. 1) and were not significantly different from those of the control group (average, 17.8 ± 0.4 mm) (Table 1). The mean concentrations of ionized calcium and soluble magnesium were 1.09 ± 0.11 meq/liter and 1.71 ± 0.14 meq/liter, respectively, in the supplemented media. These cation concentrations were not significantly different from the target concentrations of the control media.

(iii) *Pseudomonas* population response. We used 14 additional clinical isolates of *P. aeruginosa* to test the performance of cation-supplemented Mueller-Hinton agar media with a representative population of this species (Table 3). We also wished to determine whether the response of *P. aeruginosa* ATCC 27853, the recommended quality control strain (3), was typical of *P. aeruginosa* strains as a group on cation-supplemented media. Concentrations of ionized calcium and soluble magnesium in the supplemented media averaged 1.12 ± 0.09 meq/liter and 1.67 ± 0.14 meq/liter, respectively, approximately the same as the target cation concentrations of the control media (ionized calcium, 1.05 ± 0.13 meq/liter; soluble magnesium, 1.71 ± 0.15 meq/liter). Individual strains showed a range of reduction in gentamicin zone diameters from unsupplemented to supplemented media. For example, the mean differences in responses were 2.5 mm for strain UH400, 1.8 mm for strain UH805, and 1.9 mm for the standard strain, *P. aeruginosa* ATCC 27853. Overall, there was a reduction of 2.0 mm in gentamicin zone diameters for the *P. aeruginosa* population on the supplemented media: 18.3 ± 2.3 mm versus 16.3 ± 2.5 mm for the same unsupplemented lots of medium. Thus, target concentrations of cations were achieved in the supplemented media, and the population of *P. aeruginosa* responded as predicted by the control strain in gentamicin disk susceptibility tests on the supplemented media. There was, however, a significant difference of 0.8 mm ($P = 0.01$, Student's t test) in the mean of the gentamicin zone diameters from tests on the supplemented media (16.3 ± 2.5 mm) and tests performed on the control lots of media (15.5 ± 2.4 mm).

DISCUSSION

In this investigation, we demonstrated that predictable gentamicin inhibition zone sizes for *P. aeruginosa* ATCC 27853 on Mueller-Hinton agar medium could be achieved by adjustment of ionized calcium and soluble magnesium in the agar to target levels. The target levels in this case were 1.71 meq/liter for soluble magnesium and 1.05 meq/liter for ionized calcium, and they were based on the performance of Mueller-Hinton agar media from three manufacturers which gave inhibition zone sizes within the recommendation of the National Committee of Clinical Laboratory Standards. Previously, the direct effect of ionized calcium on aminoglycoside inhi-

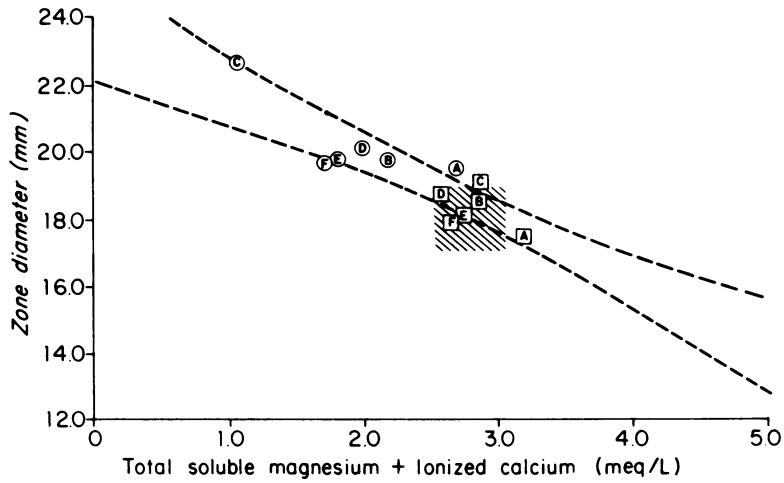

FIG. 1. *Susceptibility of P. aeruginosa ATCC 27853 to gentamicin as tested on Mueller-Hinton agars to which ionized calcium and total soluble magnesium were added to achieve final concentrations of 1.05 ± 0.13 meq/liter and 1.71 ± 0.15 meq/liter, respectively. Adjusted Mueller-Hinton agar lots are shown as follows: (A) Difco 652161; (B) Difco 655476; (C) BBL E1DHQS; (D) BBL L5DART; (E) GIBCO 870442; and (F) GIBCO 870402. Magnesium and calcium were added as gluconate salts in amounts which varied in relation to the initial concentrations of total soluble magnesium and ionized calcium (Table 1). P. aeruginosa was tested for susceptibility to gentamicin by disk diffusion on each of the prepared media. Symbols: ○, concentrations before agar manipulation of ionized calcium (0.70 ± 0.08 meq/liter) and soluble magnesium (1.21 ± 0.51 meq/liter); □, concentrations after agar manipulation of ionized calcium (1.09 ± 0.10 meq/liter) and soluble magnesium (1.71 ± 0.14 meq/liter). Area within the curves represents the 95% confidence interval for the regression equation relating the sum of ionized calcium plus soluble magnesium (milliequivalents per liter) to the gentamicin disk zone diameter. The shaded area represents the target gentamicin disk zone size of 17 to 19 mm.*

bition zone sizes for *P. aeruginosa* was demonstrated, and it was hypothesized that ionized magnesium would directly influence these tests on Mueller-Hinton agar as well (2). We present evidence here, however, that indicates that ionized magnesium has at best only a secondary influence on the inhibition zone size. It is equally clear that the ionized calcium and soluble magnesium fractions in Mueller-Hinton agar directly influence the gentamicin inhibition zone size for *P. aeruginosa*. These data suggest that the allowable range of these two fractions must become stringently defined if predictable and consistent gentamicin inhibition zone sizes are to be achieved.

Predictable Mueller-Hinton agar medium performance has been successfully achieved by defining a narrow range for these two distinct cation fractions in this study. It can be achieved by either rigorous selection of Mueller-Hinton agar or accurate adjustment of media deficient in ionized calcium or soluble magnesium or both. The choice of calcium and magnesium salts (as chloride, gluconate, or glycerophosphate) that would be best for medium supplementation was investigated because of the suggestion of Waterworth (8) that the type of salt used may influence gentamicin inhibition zone sizes for *P. aeruginosa*. Our findings indicate that addition of calcium or magnesium in the three salts tested increases but does not disturb the distribution of ions within the soluble phase of the media used in these studies and has no measurable difference in effect on the gentamicin inhibition zone diameters with any of the control strains tested. The apparent discrepancy in our findings and those cited by Waterworth (8) may be due to different media or to different anion-binding agents in the media, such as phosphates, which will influence the distribution of the cations in the previously described fractions. This aspect, however, remains to be investigated.

The objective of testing the representative nature of our observations with the control strain *P. aeruginosa* 27853 was to insure that other strains respond accordingly on manipulated media. The response of all of the strains tested to the adjustment of calcium and magnesium in Mueller-Hinton agar was in the same direction, with an average decrease in zone diameter of 2.0 mm. Comparison of the response of this population in adjusted media and control media indicates, however, that a statistical difference in inhibition zone diameters does still

TABLE 3. *Responses of different strains of P. aeruginosa to gentamicin as tested on media supplemented with ionized calcium and soluble magnesium*[a]

Pseudomonas strain	Gentamicin inhibition zone size (mm)[b] on:		
	M-H agar[c] (no supplements)	M-H agar[c] (supplemented)	M-H agar[d] (control)
ATCC 27853	20.9	19.0	18.5
Ellsworth	18.5	17.6	15.9
Mayo	21.0	19.6	18.3
UH400	12.9	10.4	9.9
UH412	13.1	10.7	10.2
UH464	21.1	19.0	18.2
UHRE	19.2	17.1	16.0
UH18	17.6	15.7	15.3
UH19	20.3	18.5	17.8
UH27	18.1	15.7	15.3
UH28	18.0	16.1	16.0
UH30	17.2	15.3	15.0
UH31	18.0	16.0	15.3
UH805	18.9	17.1	16.2
UH32	18.8	16.7	15.8
UH829	18.6	16.7	15.8
UH01	17.6	15.6	14.7

[a] $\bar{X} \pm$ standard deviation values were 18.3 ± 2.2 for unsupplemented Mueller-Hinton (M-H) agar, 16.3 ± 2.5 for supplemented M-H agar, and 15.5 ± 2.4 for control M-H agar.

[b] A 10-μg gentamicin disk was used.

[c] The average concentration of ionized calcium was 1.12 ± 0.09 meq/liter, and that of soluble magnesium was 1.67 ± 0.14 meq/liter. Media were supplemented with gluconate salt solutions. The Mueller-Hinton agar lots adjusted were Difco 652161 and 655476, BBL E1DHQS, and GIBCO 870442 and 870402.

[d] The Mueller-Hinter agar lots used were Difco 648618 and 640657, BBL G1DIAO, and GIBCO 970011.

exist. This may be reflective of a strain-related response; for example, the two strains which were the most resistant in our population appeared to be more responsive to adjustment of the ionized calcium and soluble magnesium levels in the agar. The sample size is too small, however, to make any definitive statements concerning strain differences. It is also possible that another medium component, such as zinc as postulated by Washington et al. (7), may modulate in a small but significant manner the response of *P. aeruginosa* on Mueller-Hinton agar. It should be emphasized, though, that the response of this small population of *P. aeruginosa* to manipulated and control media is not important clinically. In fact, the average inhibition zone size for both groups when the values are adjusted to the nearest millimeter is 16.0 mm.

In summary, we demonstrated that selecting a narrow range of allowable levels of ionized calcium and soluble magnesium in Mueller-Hinton agar will lead to a clinically useful and predictable gentamicin inhibition zone size for the control strain *P. aeruginosa* ATCC 27853. This can be achieved by rigorous selection of existing media or feasible adjustment of media deficient in either calcium or magnesium levels or both. Previous recommendations for calcium and magnesium in Mueller-Hinton media were based on the total amount in the agar, i.e., 1.6 to 2.8 meq/liter (20 to 35 mg/liter) and 2.4 to 5.0 meq/liter (50 to 100 mg/liter) of magnesium and calcium, respectively (5). We later determined, however, that this did not provide consistent levels of these cations in the fluid phase of the media, which apparently is the fraction that regulates the performance of the aminoglycoside on Mueller-Hinton agar (2). We now find that it is both the ionized calcium and soluble magnesium fractions which control the inhibition zone sizes. This should prove useful in improving the performance standards for *Pseudomonas* testing on Mueller-Hinton agar media.

ACKNOWLEDGMENTS

We thank Susan Swanzy for her excellent technical assistance.

This study was supported by a grant-in-aid from Difco Laboratories. B.H.M. is the recipient of Public Health Service research career development award AI-00290 from the National Institute of Allergy and Infectious Diseases.

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