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In Vitro Study of Netilmicin Compared with Other Aminoglycosides

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Netilmicin (Sch 20569) is an ethyl derivative of gentamicin C_{1a} that is active against most *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* isolates. Among 342 clinical isolates tested, all staphylococci, 92% of *Escherichia coli*, 93% of *Klebsiella pneumoniae*, and 92% of *Enterobacter* were inhibited by 0.8 μg or less of netilmicin per ml, but only 78% of *P. aeruginosa* were inhibited by 3.1 μg or less per ml. Most clinical isolates of enterococci, *Serratia marcescens*, and *Providencia* were not inhibited by 3.1 μg of netilmicin per ml. Like other aminoglycosides, the netilmicin in vitro activity was markedly influenced by the growth medium used, with activity decreased by sodium, calcium, and magnesium. Netilmicin was more active at alkaline pH. Addition of magnesium to *Pseudomonas* or *Serratia* pretreated with netilmicin produced inhibition of killing. Netilmicin was more active than gentamicin, sisomicin, tobramycin, or amikacin against *E. coli* and *K. pneumoniae*. Netilmicin inhibited growth of all gentamicin-resistant isolates of *Klebsiella* and *Citrobacter* tested, but only 73% of *E. coli; Pseudomonas* and *Providencia* were resistant to netilmicin. Most *Serratia* (95%) and indole-positive *Proteus* (83%) isolates were resistant to netilmicin but were inhibited by amikacin.

Serious infections caused by gram-negative microorganisms resistant to many of the currently available antibiotics have continued to increase (11, 12, 15). Although gentamicin has remained a highly effective antibiotic, the sporadic occurrence of strains of *Enterobacteriaceae* or *Pseudomonas* resistant to the gentamicin complex prompted us to evaluate the antimicrobial activity of netilmicin (Sch 20569), 1-*N*-ethyl sisomicin, a new semisynthetic aminoglycoside antibiotic produced by *Micromonospora inyoensis*. This compound closely resembles the C_{1a} component of the gentamicin complex.

The purpose of this study was to assess the in vitro activity of netilmicin against recent clinical, bacterial isolates, to compare the activity of netilmicin with sisomicin, gentamicin, tobramycin, and amikacin, and to determine the activity of netilmicin against clinical isolates that were resistant to gentamicin.

MATERIALS AND METHODS

Antibiotics. Netilmicin, sisomicin, and gentamicin were supplied by Schering Corp. Tobramycin was a gift from Eli Lilly & Co., and amikacin was a gift from Bristol Laboratories. Fresh dilutions of all the aminoglycoside antibiotics were prepared daily in sterile medium or distilled water. Bacterial isolates were obtained from patients hospitalized at the Columbia Presbyterian Medical Center during the past 2 years. The isolates came from sputum, blood, and urine specimens and did not represent the same strains insofar as could be determined by general antibiograms and bacteriocin typing.

Susceptibility tests. The antimicrobial activity was measured by the broth dilution method as previously described (6). Serial twofold dilutions of antibiotics in Mueller-Hinton broth (BBL) were used. An inoculum of 0.5 ml containing 10^5 cells (colony-forming units [CFU]) from a diluted overnight culture was added to 0.5 ml of antibiotic solution. Cultures were incubated at 35°C for 18 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration that inhibited development of visible turbidity. The minimum bactericidal concentration (MBC) was determined by plating 0.01 ml from clear tubes onto agar. MIC values were also determined by the agar dilution method (6). A 100-fold dilution of an overnight culture was applied with an inocula replicator. The MIC was taken as the highest concentration of antibiotic on which there was no visible growth or less than five colonies.

The effect of growth medium on the activity of netilmicin was determined with Trypticase soy (BBL), brain heart infusion (BBL), and nutrient broth (BBL). Concentrations of ions were determined by standard chemical assays, using flame photometry and/or atomic absorption spectrophotometry.

RESULTS

Antimicrobial activity of netilmicin. The in vitro activity of netilmicin against 342 gram-positive and gram-negative organisms is summarized in Table 1. All isolates of *Citrobacter* and *Staphylococcus aureus* were inhibited at a concentration of 0.8 μg of netilmicin per ml. Of the *Escherichia coli, Klebsiella, Enterobacter,* and *Salmonella* isolates tested, more than 90% were inhibited by 0.8 μg/ml. At a concentration of 3.1 μg/ml, 91% of *Proteus mirabilis*, 81% of indole-positive *Proteus*, and 75% of *Acinetobacter* were inhibited. There were 51% of the strains of *P. aeruginosa* inhibited by 0.8 μg of netilmicin per ml and 78% inhibited by 3.1 μg/ml. Markedly resistant strains (≥12.5 μg/ml) were rare. A number of *Serratia marcescens, Providencia*, and enterococcal isolates had netilmicin MIC values above 6.3 μg/ml.

Effect of growth medium. The size of the initial inoculum of most organisms tested had little effect on the netilmicin MIC values determined by the broth dilution method (Table 2). However, *Serratia* isolates showed a two- to eightfold increase in MIC values when the inoculum size was increased from 10^3 to 10^7 CFU.

The effect of different medium and pH of the medium used to determine the activity of netilmicin is shown in Table 3. The netilmicin MIC values determined for *Pseudomonas, E. coli, Klebsiella,* and *Serratia* were lowest in nutrient broth among the four media tested. Netilmicin was also more active at pH 8 than at pH 6 regardless of the medium used.

A comparison of MIC and MBC values of netilmicin showed that the MBC values were only slightly higher than its MIC. MIC values determined by both agar dilution and broth dilution were similar, except for *Pseudomonas* and *Serratia* isolates (Table 4). A 2- to 16-fold increase in MIC value was observed in Mueller-Hinton agar as compared with that in Mueller-Hinton broth for 13 of 22 strains of *P. aeruginosa* tested. The MIC values of netilmicin were significantly higher in nutrient broth with added cations than those in nutrient broth, even at the physiological concentration of calcium, magnesium, and sodium (Table 5). Magnesium had the greatest inhibitory effects at an equimolar concentration among the cations tested. This is also illustrated in Fig. 1. At the concentration of 2 mM magnesium, the netilmicin inhibition of *Serratia* could be reversed within 1 h, whereas it took 10 mM calcium to

TABLE 2. *Effect of inoculum size on the activity of netilmicin (Sch 20569)*[a]

Organism	MIC (μg/ml) at:		
	10^3 CFU	10^5 CFU	10^7 CFU
E. coli 2826	0.8	3.1	3.1
E. coli 2175	1.6	3.1	3.1
P. aeruginosa 2182	0.2	0.4	0.4
P. aeruginosa 2716	0.2	0.2	0.4
P. aeruginosa 2699	0.4	0.8	1.6
P. aeruginosa 2632	1.6	1.6	1.6
P. aeruginosa 2139	1.6	1.6	3.1
K. pneumoniae 2920	0.2	0.4	0.8
K. pneumoniae 2933	0.1	0.2	0.4
S. marcescens 2944	3.1	3.1	6.3
S. marcescens 2744	25	25	100
S. marcescens 2569	12.5	25	25
S. marcescens 2327	1.6	1.6	6.3

[a] Mueller-Hinton broth was used to determine MIC.

TABLE 1. *Cumulative percentage of isolates susceptible to netilmicin (Sch 20569)*[a]

Organism	No. of isolates	Susceptible isolates (%) at an MIC (μg/ml) of:											
		0.025	0.05	0.1	0.2	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100
S. aureus	20		4	40	68		100							
Enterococci	32								12	62	88	100		
E. coli	27				26	81	92			100				
Shigella	14				12		78	100						
K. pneumoniae	29				63	89	93		96	100				
Enterobacter	25				28	80	92		96	100				
S. marcescens	29					4		10	17	27	64	78	92	100
Salmonella	25				8	72	96	100						
Citrobacter	20	15			35	60	100							
Proteus, indole positive	26				18	73		76	81	92	100			
P. mirabilis	29				3	21	31	68	91			95	100	
Providencia	15					7		13	26	86	93	100		
P. aeruginosa	41				10	32	51	74	78	87	96		100	
Acinetobacter	10				10	30	40	70		80		90	100	

[a] MICs were determined in Mueller-Hinton agar.

TABLE 3. *Effect of medium and its pH on the activity of netilmicin (Sch 20569)*

Organism	MIC and MBC (μg/ml)[a]											
	Brain heart infusion			Mueller-Hinton broth			Trypticase soy broth			Nutrient broth		
	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8
E. coli 2826	3.1	3.1	0.8	3.1	0.8	0.4	3.1	3.1	0.2	0.025	0.025	<0.025
	(3.1)	(3.1)	(3.1)	(3.1)	(3.1)	(0.4)	(3.1)	(6.3)	(0.2)	(0.025)	(0.025)	(<0.025)
P. aeruginosa 2182	0.8	0.8	0.8	0.4	0.4	0.4	1.6	1.6	0.8	0.05	0.05	<0.025
	(3.1)	(0.8)	(1.6)	(0.8)	(0.4)	(0.8)	(3.1)	(3.1)	(0.8)	(0.05)	(0.05)	(0.025)
K. pneumoniae 2920	1.6	0.8	0.8	1.6	0.4	0.2	3.1	6.3	0.4	<0.025	<0.025	<0.025
	(3.1)	(0.8)	(0.8)	(1.6)	(0.4)	(0.2)	(3.1)	(6.3)	(0.8)	(<0.025)	(0.025)	(0.025)
S. marcescens 2744	50	50	50	100	25	25	25	25	25	3.1	0.8	0.4
	(50)	(100)	(50)	(100)	(50)	(100)	(100)	(25)	(100)	(3.1)	(3.1)	(0.8)

[a] Numbers in parentheses indicate MBCs.

TABLE 4. *Effect of broth dilution and agar dilution method on the activity of netilmicin (Sch 20569)*

Organism	MIC (μg/ml) in Mueller-Hinton agar	Mueller-Hinton broth	
		MIC (μg/ml)	MBC (μg/ml)
E. coli 2826	1.6	1.6	3.1
E. coli 2938	6.3	6.3	12.5
E. coli 2939	6.3	3.1	3.1
K. pneumoniae 2933	0.2	0.1	
K. pneumoniae 2920	0.8	0.2	0.4
K. pneumoniae 2940	0.4	0.2	
S. marcescens 2744	25	12.5	25
S. marcescens 2944	25	6.3	
P. vulgaris 2924	0.4	0.4	
P. mirabilis 2339	12.5	12.5	
P. aeruginosa 2182	0.8	0.2	0.8
P. aeruginosa 2923	12.5	3.1	12.5
P. aeruginosa 2968	6.3	6.3	25
P. aeruginosa 2142	12.5	3.1	3.1
P. alcaligenes 2942	50	50	50
P. maltophilia 2941	12.5	12.5	25
P. maltophilia 2975	50	25	50

antagonize netilmicin growth inhibition of *Serratia*. The addition of calcium or magnesium within 1 h to cells of *Pseudomonas* or *Serratia* pretreated with netilmicin would halt normal killing and allow regrowth. The addition of horse blood had little effect on the activity of netilmicin against *P. aeruginosa*, *K. pneumoniae*, and *E. coli* (Table 6).

Comparative activity of netilmicin with gentamicin, sisomicin, tobramycin, and amikacin. The comparative in vitro activity of netilmicin, amikacin, sisomicin, tobramycin, and gentamicin against 342 clinical isolates determined by the agar dilution method is shown in Fig. 2 through 7. The activity of netilmicin closely paralleled that of gentamicin, sisomicin, and tobramycin. Netilmicin was the most active of the aminoglycosides tested against *Klebsiella* and *E. coli*. Netilmicin was twofold more active against *E. coli* than were gentamicin and sisomicin, which were in turn more active than tobramycin and amikacin. Sisomicin, tobramycin, and gentamicin were equally

TABLE 5. *Effect of cations on the activity of netilmicin (Sch 20569)*

Cation[a]	Concn of added salt (mM)	MIC (μg/ml)[b]			
		P. aeruginosa	*K. pneumoniae*	*S. marcescens*	*E. coli*
Calcium	0	0.06	0.03	0.5	0.03
	0.025	0.06	0.03	1	0.06
	0.5	0.06	0.03	1	0.06
	1.0	0.25	0.03	2	0.12
	2.0	1	0.03	4	0.12
Magnesium	0.1	0.06	0.03	0.25	1
	1	1	0.12	1	8
	2	4	1	8	64
Sodium	2	0.06	0.03	0.03	0.5
	10	0.06	0.06	0.12	2.0
	50	0.06	0.25	0.5	8.0
	100	0.12	0.5	1	64

[a] Calcium was added as $CaCl_2$, magnesium as $MgSO_4$, and sodium as NaCl.

[b] MIC values were determined in nutrient broth that has an Mg^{2+} of 0.18 mM, a Ca^{2+} of 0.02 mM, and an Na^+ of 9.8 mM.

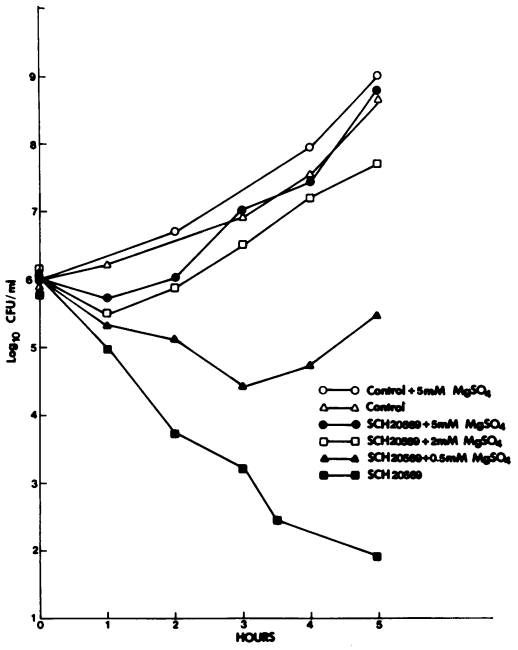

FIG. 1. *Rate of killing of S. marcescens 2944 in nutrient broth (BBL) containing 2 μg of netilmicin (Sch 20569) per ml and $MgSO_4$ at the concentration shown.*

TABLE 6. *Effect of horse blood on the activity of netilmicin (Sch 20569)*[a]

Organism	No. of isolates	Horse blood added (%)	MIC (μg/ml)	
			Range	Median
E. coli	24	0	0.31-2.5	0.47
		1	0.31-2.5	0.40
		5	0.31-2.5	0.46
		10	0.31-2.5	0.32
K. pneumoniae	20	0	0.31-2.5	0.62
		1	0.31-2.5	0.70
		5	0.31-2.5	0.45
		10	0.31-2.5	0.45
P. aeruginosa	22	0	1.25-40	10.10
		1	1.25-40	7.50
		5	1.25-50	7.50
		10	1.25-40	9.0

[a] MICs were determined by agar dilution method on Mueller-Hinton agar containing horse blood as noted.

active against *Klebsiella* and less active than netilmicin, which in turn was about threefold more active than amikacin at lower concentrations. On the other hand, netilmicin was the least active against *Providencia*. Amikacin was consistently the least active agent against aminoglycoside-susceptible isolates, although it was most active against *Providencia* and *Serratia*. Tobramycin was slightly more active than

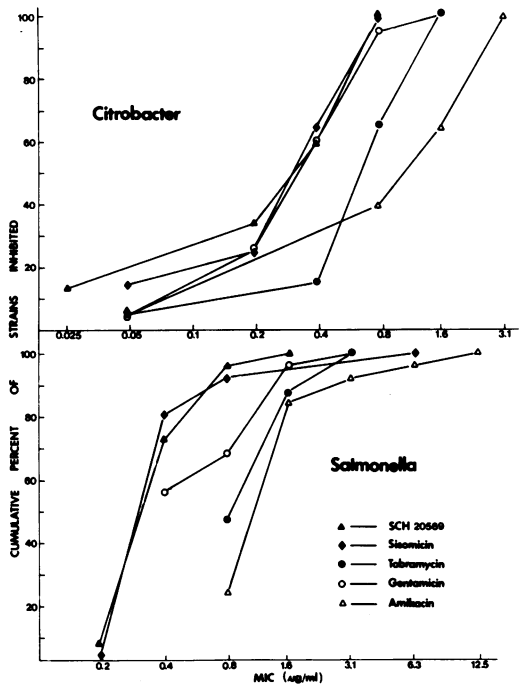

FIG. 2. *Comparative activity of aminoglycoside antibiotics against Citrobacter (20 isolates) and Salmonella (25 isolates). Tested by agar dilution method. Scale is logarithmic. Sch 20569, Netilmicin.*

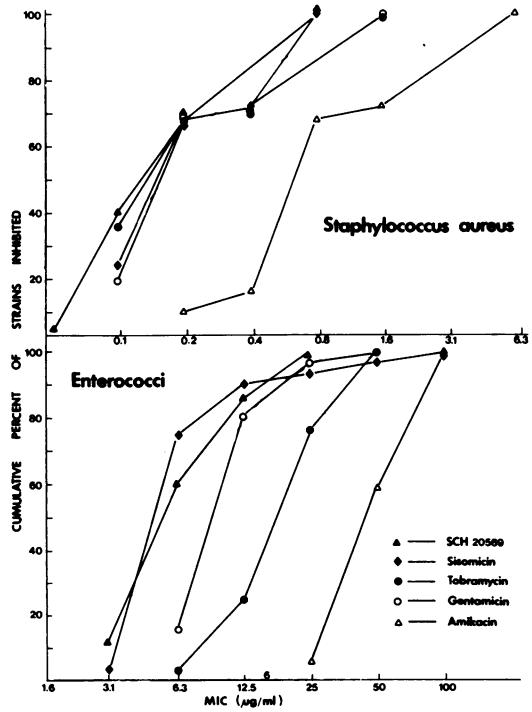

FIG. 3. *Comparative activity of aminoglycoside antibiotics against S. aureus (20 isolates) and enterococci (32 isolates). Sch 20569, Netilmicin.*

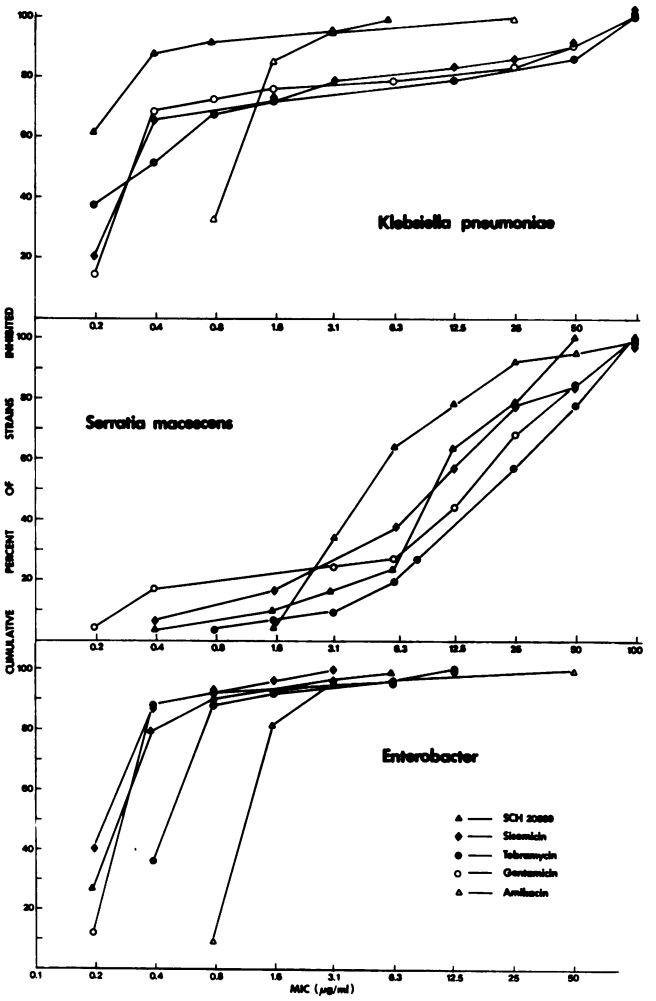

FIG. 4. *Comparative activity of aminoglycoside antibiotics against Enterobacter (25 isolates), S. marcescens (29 isolates), and K. pneumoniae (29 isolates). Sch 20569, Netilmicin.*

sisomicin and two- to fourfold more active than gentamicin against *Pseudomonas*. Netilmicin was much less active against *Pseudomonas* than the aforementioned agents, but more active than amikacin.

Activity of netilmicin against gentamicin-resistant isolates. Table 7 enumerates the activity of netilmicin against 68 gentamicin-resistant isolates. The majority of isolates of *E. coli, Klebsiella, Enterobacter,* and *Citrobacter* that had gentamicin MIC values of 12.5 μg or greater per ml were susceptible to 3.1 μg or less of netilmicin per ml. Most gentamicin-resistant *S. marcescens, Providencia, P. rettgeri,* and *P. aeruginosa* had netilmicin MIC values of 12.5 μg or greater per ml.

A comparison of the activity of gentamicin, netilmicin, tobramycin, sisomicin, and amikacin demonstrated that gentamicin-resistant *S. marcescens* were also resistant to tobramycin and sisomicin, whereas all but one were susceptible to concentrations of amikacin that could be achieved in humans. Gentamicin-resistant *K. pneumoniae* were resistant to sisomicin and tobramycin, but very susceptible to netilmicin, which is three times more active than amikacin. *Enterobacter* resistant to gentamicin were usually resistant to tobramycin and sisomicin, with rare exceptions. A similar situation was encountered with *E. coli*. Some gentamicin-resistant *P. aeruginosa* isolates were susceptible to tobramycin and to sisomicin, and all but

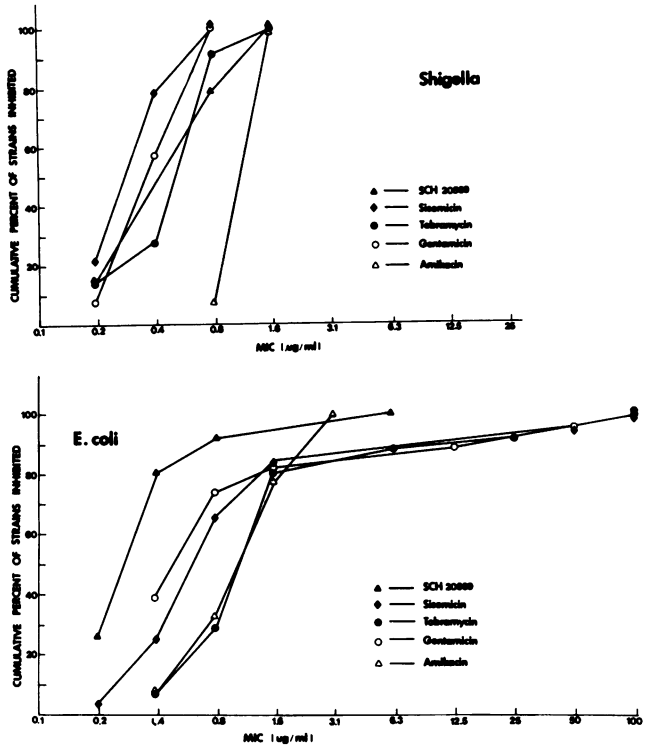

FIG. 5. *Comparative activity of aminoglycoside antibiotics against Shigella (14 isolates) and E. coli (27 isolates). Sch 20569, Netilmicin.*

TABLE 7. *Comparative activity of netilmicin (Sch 20569), amikacin, sisomicin, and tobramycin against gentamicin-resistant organisms*

Organism	No. of strains	MIC (μg/ml)									
		Gentamicin		Netilmicin		Tobramycin		Sisomicin		Amikacin	
		Range	Median	Range	Median	Range	Median	Range	Median	Range	Median
S. marcescens	22	6.3->100	37.5	3.1->100	32.5	1.6->100	40	0.4->100	21.3	3.1-50	10
K. pneumoniae	11	25->100	65	0.2-6.3	0.8	3.1->100	50	12.5->100	60	1.6-6.3	2.6
Providencia 2943		12.5		25		6.3		3.1		1.6	
Providencia 748	2	6.3		12.5		6.3		3.1		1.6	
Enterobacter	3	6.3-50	16.5	3.1-12.5	6.3	6.3-50	16.5	1.6-50	16.5	0.8-50	6.3
Citrobacter 3007	1	50		0.4		50		50		1.6	
P. mirabilis 2576	1	50		50		50		50		50	
P. rettgeri	4	6.3-12.5	7.8	3.1-25	12.5	3.1-6.3	4.7	1.6-6.3	3.1	0.8-1.6	1
P. morganii	1	6.3		6.3		12.5		6.3		1.6	
P. vulgaris	1	6.3		25		12.5		6.3		3.1	
E. coli	11	12.5->100	40	0.4-12.5	4.2	1.6->100	40	0.8->100	35	0.4-12.5	2.1
P. aeruginosa	6	6.3->100	18.8	12.5-50	8.1	0.8->100	8.1	1.6-50	4.6	1.6-50	9.3
P. maltophilia	4	12.5-50	37.5	25-50	31.3	12.5-50	31.3	1.6-50	18.8	50-50	50
P. alcaligenes	1	100		>100		12.5		50		50	

one were susceptible to amikacin. Amikacin was the agent active against the largest number of gentamicin-resistant isolates of all organisms tested.

DISCUSSION

Netilmicin, an ethyl derivative of gentamicin C_{1a}, has excellent activity against the majority

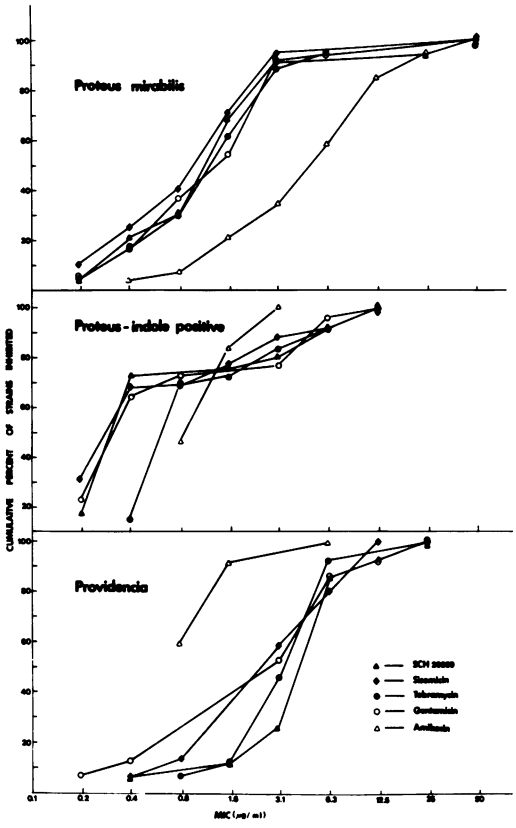

FIG. 6. *Comparative activity of aminoglycoside antibiotics against P. mirabilis (29 isolates), indole-positive Proteus (26 isolates), and Providencia (15 isolates). Sch 20569, Netilmicin.*

of gram-negative bacteria and *S. aureus*. Most of the isolates tested were inhibited by netilmicin at the concentration achievable in human blood (S. Sate, personal communication). Several reports have documented that factors such as inoculum size (6), presence of serum (5), and different medium and the pH of the medium (6), as well as cation content of the medium, greatly influence the activity of aminoglycoside antibiotics (7, 10, 14). These factors were shown to affect the MIC values of netilmicin.

Netilmicin was most active in nutrient broth which contains less magnesium and calcium than that encountered in the physiological situation. Netilmicin, like most other aminoglycosides, was more active in an alkaline medium (6, 16). The low MIC of netilmicin in nutrient and alkaline medium may be due to more active netilmicin accumulation in cells by active transport (2). Zimelis and Jackson (18) showed that the calcium and magnesium antagonism is species specific against *P. aeruginosa*. In this investigation, we observed significant changes in the MIC of netilmicin not only against *P. aeruginosa* but also against *Klebsiella, E. coli,* and *S. marcescens* brought about by calcium and magnesium. The reason may be due to the different medium utilized.

In agreement with other reports (3, 9), tobramycin was found to be the most active aminoglycoside against *P. aeruginosa*, and amikacin was constantly the least active aminoglycoside on a basis of micrograms per milliliter. Netilmicin was threefold less active than gentamicin against *Pseudomonas* isolates. Rahal et al. (13) recently found that netilmicin, gentamicin, and amikacin exhibited similar activity against *Pseudomonas*, in contrast to the data of this paper. Our median MIC values of netilmicin, amikacin, and gentamicin are 0.9, 2.25, and 0.38 μg/ml, respectively. The difference in results may be caused by the more resistant organisms in the Rahal et al. (13) study. Furthermore, in contrast to the data of Rahal et al. (13), we found that *Citrobacter* were equally susceptible to gentamicin, sisomicin, and netilmicin. The reason for the difference in our results may be that we have not encountered significant aminoglycoside resistance in our institution.

Amikacin was the most active agent against gentamicin-resistant *S. marcescens*. The mean MIC values against seven gentamicin-susceptible *Serratia* strains were 1.14 μg of gentamicin per ml, 4.95 μg of sisomicin per ml, 34.5 μg of netilmicin per ml, 7.36 μg of amikacin per ml, and 23.3 μg of tobramycin per ml. Netilmicin was active against *E. coli, Klebsiella,* and *Enterobacter* which were resistant to gentamicin. This was also true of amikacin but not of sisomicin and tobramycin.

The in vitro disadvantage of amikacin as compared with the other amino-glycoside antibiotics appears to be offset by the evidence that it achieves significantly higher serum levels in animals and humans (4) and it was active against netilmicin-resistant or other aminoglycoside-resistant organisms, as shown in this study which was in agreement with other reports (12, 13, 17).

Aminoglycoside resistance mediated by enzymatic acetylation, adenylylation, and phosphorylation has been well documented (1, 8) and undoubtedly explains the resistance of isolates to netilmicin. We currently are determining the enzymatic inactivating patterns of the organisms that are resistant to netilmicin.

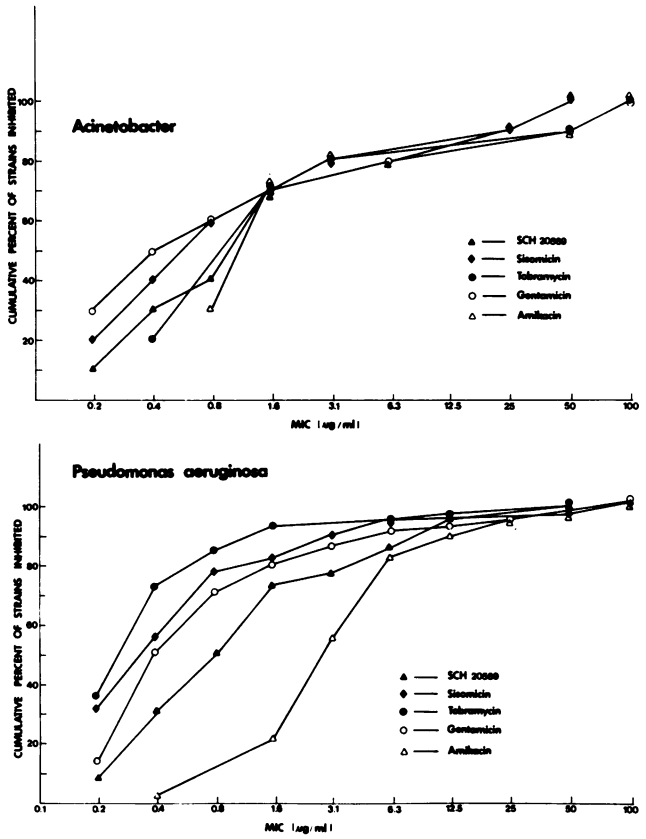

FIG. 7. *Comparative activity of aminoglycoside antibiotics against Acinetobacter (10 isolates) and P. aeruginosa (41 isolates). Sch 20569, Netilmicin.*

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