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Comparison Between Agar and Broth Minimum Inhibitory Concentrations of Cefamandole, Cefoxitin, and Cefuroxime

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The in vitro susceptibility to cefamandole, cefoxitin, and cefuroxime of clinical isolates of *Streptococcus faecalis, Klebsiella,* and indole-positive *Proteus* was assayed using minimum inhibitory concentration (MIC) determinations in broth and on solid media. It could be demonstrated that the agar dilution MICs obtained when *S. faecalis* was tested against cefuroxime and when indole-positive *Proteus* strains were tested against cefamandole tended to be considerably lower than those obtained with the broth dilution technique. The *Klebsiella* strains tested did not show any major differences with regard to MICs in broth or on solid media. Using an animal experimental infection model it could be demonstrated that with indole-positive *Proteus* the higher broth MIC correlated better to the observed data than the lower agar MIC when a β-lactamase-producing strain was tested. The data obtained indicated that the β-lactamase of the indole-positive *Proteus* strain was inducible. The results of the study gave evidence for a risk of false susceptibility of some bacterial species against cefamandole when agar techniques, e.g. paper disk diffusion, are used. For cefuroxime, the same phenomenon can be expected with *S. faecalis* and to some extent with indole-positive *Proteus.* In this study, cefoxitin seemed considerably less affected by the technique used for susceptibility testing.

In vitro antibiotic susceptibility of bacteria can be assayed by various techniques. In routine laboratory work, the paper disk diffusion methods (1, 4) still dominate. These techniques are based on relations between the diameters of the inhibition zones around antibiotic-containing paper disks and the minimum inhibitory concentrations (MICs) of the same strains, usually assayed by agar dilution. Thus, the MICs obtained when solid media are used are of vital importance for the outcome of routine sensitivity tests.

MICs can also be determined by diluting antibiotics in nutrient broth instead of solid medium. Several authors have reported broth MICs for certain cephalosporins to differ considerably from those obtained with agar dilution (3, 11). Thus, it has recently been reported that certain strains of *Enterobacter* and indole-positive *Proteus* show higher MICs in broth than on solid medium (2, 6) when tested against cefamandole. These observations initiated the present study on the correlation between the size of the inhibition zones around antibiotic-containing disks and MICs in broth and on solid medium for cefamandole, cefoxitin, and cefuroxime when tested against clinical bacterial isolates. Moreover, the in vitro and in vivo activity of cefamandole on two strains of indole-positive *Proteus* was studied with respect to β-lactamase production of the two strains.

MATERIALS AND METHODS

Bacterial strains. All strains were clinical isolates identified according to conventional methods. They were all part of a previously published study on the susceptibility of a large number of pathogens to cefamandole, cefuroxime, cefoxitin, and cephalothin (2).

Antibiotics. Cefuroxime was obtained from Glaxo Research Laboratories, Greenford, Middlesex, England, cefamandole came from Eli Lilly, Indianapolis, Ind., and cefoxitin was from Merck Sharp & Dohme Research Laboratories, Rahway, N.J. All substances were obtained as dry powder with known potency.

MIC determinations. (i) Solid medium. In all experiments, PDM medium (AB Biodisk, Solna, Sweden) was used. The antibiotics were added as freshly prepared solutions in physiological saline giving final concentrations ranging from 128 to 0.25 μg/ml in twofold dilutions. The inocula used were 4-h broth cultures diluted to contain 5×10^5 colony-forming units (cfu) per ml, and about 5×10^2 cfu of each strain was transferred to the plates with a modification of the Steers replicator. When PDM agar plates were flooded with the inocula used, the resulting bacterial growth was "just short of confluent," i.e., the inocula recommended for agar dilution MICs and for disk diffusion

tests when constructing regression lines (4). The MICs were read as the lowest concentrations completely inhibiting bacterial growth after incubation at 37°C for 20 h.

(ii) Fluid medium. For MIC determinations in fluid medium, a Dynatech 2000 dispensor and inoculator were used. Microtiter plates were filled with 0.1-ml samples of the antibiotic dilutions in PDM broth. The concentration of each antibiotic ranged from 128 to 0.25 μg/ml in twofold dilutions. The plates were kept at −20°C for a maximum period of 2 weeks prior to use. About 5×10^4 cfu of 4-h broth cultures was transferred to each well, corresponding to an inoculum of 5×10^5 cfu/ml, i.e., the inoculum recommended for broth dilution MIC determinations (4, 12). All antibiotics were studied with the same bacterial suspension of each strain. MICs were read as the lowest concentration inhibiting visible growth after incubation for 20 h at 37°C.

Disk diffusion tests. All strains were submitted to disk diffusion tests on PDM agar (4). When flooding the plates, the same inocula as for the agar dilution MIC determinations were used, giving the recommended just-short-of-confluent growth (4, 5). The 30-μg disks were obtained from the manufacturers of the antibiotics.

Growth curve studies in the presence of cefamandole. Eighteen-hour cultures of two strains of indole-positive *Proteus morganii* (P56 and P68) were added to PDM broth to give an inoculum of 10^7 cfu/ml and a final cefamandole concentration of 25 μg/ml. Viable counts were performed immediately after inoculation and after 2, 4, and 24 h of incubation on blood agar plates flooded with an amount of penicillinase (Penicillinase, Lövens Kemiska Fabrik, Denmark) sufficiently high to destroy cefamandole.

When sampling was performed after 24 h, samples were also plated on blood agar plates containing 5 μg of cefamandole per ml. Individual colonies growing on these plates were checked for β-lactamase production by the chromogenic cephalosporin 87/312 (7).

In vivo experiments. The two indole-positive *Proteus* strains used in the above experiment were used for inoculating the fluid of steel-net cages implanted subcutaneously in two rabbits. The steel-net cage technique has been described previously (9). The tissue cages had been implanted 4 weeks prior to inoculation. Treatment with cefamandole (50 mg/kg of body weight per dose) was started 48 h after inoculation of the cages when the viable count in the cages had increased from 10^6 to about 10^7 cfu/ml. The first injection was given intravenously and the remaining injections were given intramuscularly every 8 h for 7 days. Samples of blood and tissue cage fluid were drawn 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h after the first intravenous injection and on day 3 after an intramuscular injection.

The cefamandole activity was determined microbiologically using an agar well diffusion method. At sampling, 2 parts of infected tissue cage fluid were immediately mixed with 1 part of 70 mM potassium iodine solution to interrupt enzymatic destruction of cefamandole. When assaying these samples, the standard series were treated similarly. The amount of iodine solution necessary had been determined in in vitro experiments. Samples of tissue cage fluid were drawn each morning prior to the cefamandole injections, and the number of cfu was assayed on blood agar plates.

There were five steel-net cages in each rabbit. Two cages in each rabbit were inoculated with each strain, and the fifth cage served as uninoculated control.

Determination of β-lactamase activity. The *Proteus* strains used for MIC determinations were heavily seeded on blood agar plates on which 30-μg cefamandole disks were placed. After incubation at 37°C for 24 h, colonies were picked from the brims of the zones of inhibition and suspended in 5 ml of cefamandole solution (30 μg/ml in 100 mM phosphate buffer, pH 7) to a density of about 10^9 cfu/ml. These suspensions were incubated at 37°C for 4 h. The supernatants obtained after centrifugation at 25,000 × *g* for 15 min were checked for remaining cefamandole by means of absorption measurement at 268 nm in a Beckman BD spectrophotometer.

RESULTS

The MICs obtained on solid and in fluid medium were compared for strains of indole-positive *Proteus* and *Klebsiella* when tested against cefamandole, cefoxitin, and cefuroxime and for *Streptococcus faecalis* when tested against cefamandole and cefuroxime (Fig. 1). Almost all strains of *S. faecalis* were inhibited by 32 μg or less of cefuroxime per ml on solid medium. In fluid medium the strains were less susceptible, and the correlation between MICs in fluid and on solid medium was very poor. The MICs of cefamandole against these strains were less affected by the technique used ($r = 0.675$). Cefoxitin was not included since all strains had MICs of 128 μg/ml or more.

Cefoxitin had about the same MICs against the strains of indole-positive *Proteus* regardless of the technique used ($r = 0.851$). When tested against cefamandole, several strains showed a dramatic increase in MICs when tested in broth compared to MICs observed on solid medium, and the correlation between these values was poor ($r = 0.530$). Some of the indole-positive strains of *Proteus* showed higher MICs in fluid medium than on solid medium when tested against cefuroxime ($r = 0.723$). The strains of *Klebsiella* had about the same MICs both in fluid and on solid medium, and the correlation between these values seemed acceptable for all drugs (cefamandole $r = 0.671$, cefoxitin $r = 0.886$, and cefuroxime $r = 0.839$).

The relationship between the zones of inhibition around 30-μg disks and the MICs registered on agar and in broth can be seen from Fig. 2A and B.

The constructed regression lines showed that for the *Klebsiella* strains a given zone size for any of the three antibiotics would correspond to similar broth and agar MICs. The same obser-

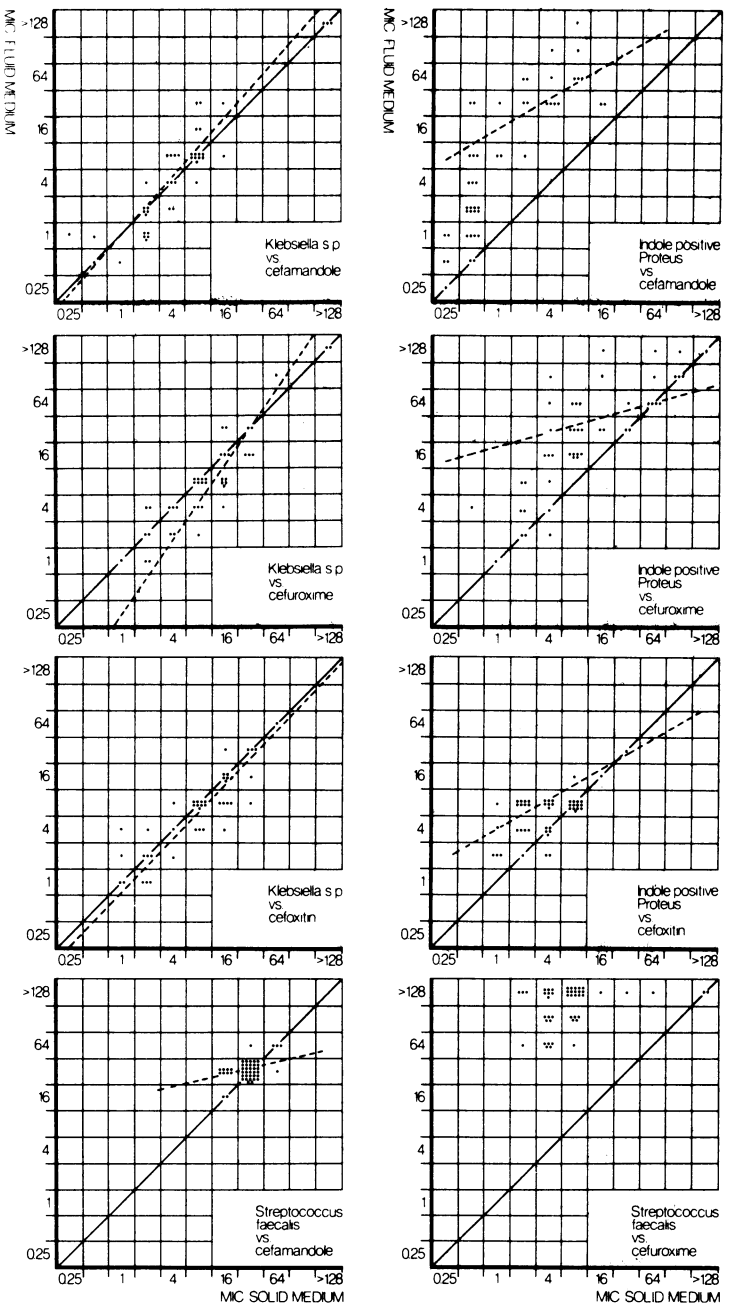

FIG. 1. *Comparison between agar and broth MICs (micrograms per milliliter). Klebsiella (43 strains), indole-positive Proteus (48 strains), and S. faecalis (47 strains) were tested against cefamandole, cefuroxime, and cefoxitin. Each dot represents one strain. (- - -) Observed regression line; (——) ideal regression line.*

vation was made with cefoxitin against indole-positive *Proteus* and cefamandole against *S. faecalis*. For other combinations of drug and bacterial species, e.g., cefamandole against indole-positive *Proteus* and cefuroxime against indole-positive *Proteus* and *S. faecalis*, it was noted that the regression lines for fluid and solid media were discordant. Furthermore, it was noted that a given zone size corresponded to a wider range of broth MICs than agar MICs.

Strains P56 and P68 were both inhibited by 0.5 μg of cefamandole per ml when tested on solid medium. However, when tested in fluid medium, strain P56, not producing β-lactamase, still had an MIC of 0.5 μg/ml, whereas strain P68, producing β-lactamase, had an MIC of 32 μg/ml, i.e., a 64-fold increase as compared to the MIC on solid medium. With the disk diffusion method both strains had a zone diameter of 28 mm. When these strains were exposed to cefamandole in fluid medium in a concentration 50 times higher than the MIC on solid medium, strain P56 showed a continuous decline in viable count during the 24-h observation period. Strain P68, on the other hand, showed an initial decline in viable count followed by regrowth (Fig. 3).

Colonies from strain P68 growing on plates containing cefamandole were checked for β-lactamase production. About 80% of the colonies tested produced β-lactamase.

The in vivo experiments showed that the concentrations of cefamandole in the uninoculated cages were reproducible after the two test injections and also between the two individual rabbits. In the cages inoculated with strain P56, the cefamandole concentrations were lower than in the uninoculated control cage (Fig. 4A and B). The number of viable bacteria was slightly decreased in the cages infected with strain P56 (Fig. 5). In the cages inoculated with strain P68, cefamandole could be demonstrated only in the beginning of the observation period and not on day 3 (Fig. 4A and B). There was no reduction in viable count in the cages inoculated with strain P68 (Fig. 5).

Of the 48 investigated *Proteus* strains, 28 were found to produce β-lactamase capable of breaking down cefamandole (Table 1). As can be seen, discrepant broth and agar MICs were observed not only in strains producing β-lactamase but also in strains lacking this ability.

DISCUSSION

It was evident from this study that agar and broth MICs correlate poorly for certain combinations of bacterial species and cephalosporin antibiotics, e.g., cefamandole versus indole-positive *Proteus* and cefuroxime versus *S. faecalis*. It was also found that a given zone size around an antibiotic-containing disk for these combinations of drug and bacterial species corresponded to divergent broth and agar MICs. On the other hand, for other combinations of drug and bacterial species the correlation between broth and agar MICs was good and a given zone size corresponded to similar broth and agar MICs.

The number of bacteria exposed to antibiotic activity is larger in the recommended broth dilution method (4, 12) than in the agar dilution method (4) for MIC determinations. Repeated trials showed that the number of cfu per spot transferred from a suspension giving just-short-of-confluent growth was about 5×10^2 for the fast-growing bacteria used in this study. This number should be compared to the number of cfu used in the broth MIC determinations. It should also be noted that the inocula recommended in both methods are small compared to the number of bacteria said to be present in some infections (10). With the broth technique, the possibility increased to include cells with decreased susceptibility to the antibiotic. Thus, the observed discrepancies might be explained by heterogenicity of the inocula.

The majority of the indole-positive *Proteus* strains could destroy cefamandole, indicating production of extracellular cephalosporinases. It cannot be excluded, however, that some strains produce low amounts of intracellular cephalosporinases not detected by the technique used. In accordance with previous observations on cefamandole-resistant *Enterobacter* strains (6), there was no correlation between the ability to hydrolyze cefamandole and discrepant agar and broth MICs. The finding that some resistant colonies from strain P68 produced β-lactamase whereas others did not demonstrates that in the same bacterial strain more than one factor may be involved in antibiotic resistance.

Of the compounds tested, cefamandole is the least stable to the action of β-lactamases and cefoxitin the most stable, and cefuroxime takes an intermediate position (8). The stability of cefoxitin to β-lactamases is a most plausible explanation of the fact that the MICs for indole-positive *Proteus* were little affected by the technique used for sensitivity testing. Regarding the discrepancies observed when *S. faecalis* was tested against cefuroxime, other resistance factors must have been involved.

The observed differences could be clinically relevant. If broth MICs more adequately reflect the in vivo efficacy of an antibiotic than agar MICs, the MIC value registered in broth should be regarded as clinically relevant. It has been found that agar MICs are poor in predicting the outcome of experimental infections in animal models for certain antibiotics (A. K. Miller, E. Celozzi, B. A. Pelak, J. Birnbaum, and E. O. Stapley, 10th International Congress of Chemotherapy, Zurich, abstr. no. 26, 1977). The explanation for this observation might be discrepant broth and agar MICs. The results obtained with the two *Proteus* strains indicate that the broth

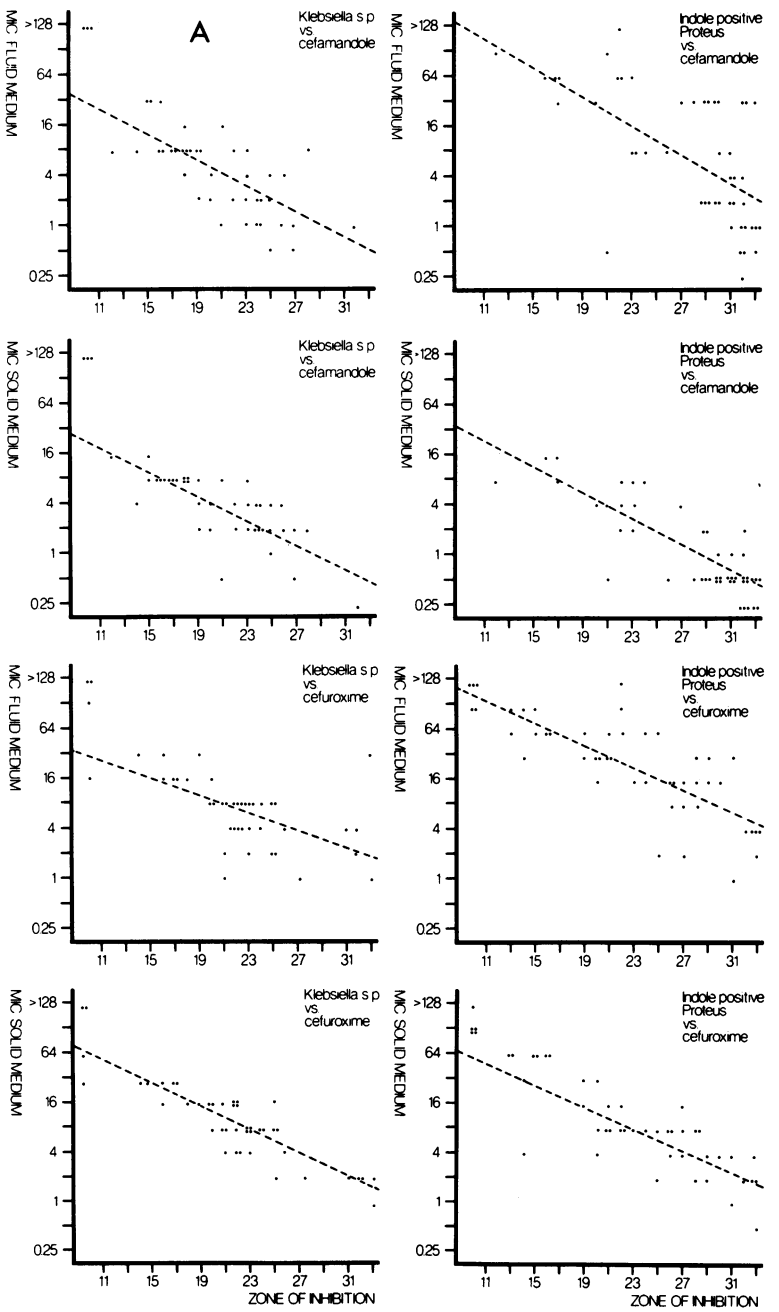

FIG. 2. *Comparison between inhibition zone diameter (millimeters) and broth and agar MICs (micrograms per milliliter). Klebsiella (43 strains), indole-positive Proteus (48 strains), and S. faecalis (47 strains) were tested against cefamandole, cefuroxime, and cefoxitin. Each dot represents one strain. (- - -) Observed regression line.*

MIC predicted the antibiotic concentrations attained in the in vivo experiments more correctly than the agar MIC for the β-lactamase-producing strain P68. This strain produced β-lactamase despite a low agar MIC and a zone size of 28 mm with the disk diffusion method and destroyed cefamandole when infections in tissue chamber fluid were studied. In correlation with that find-

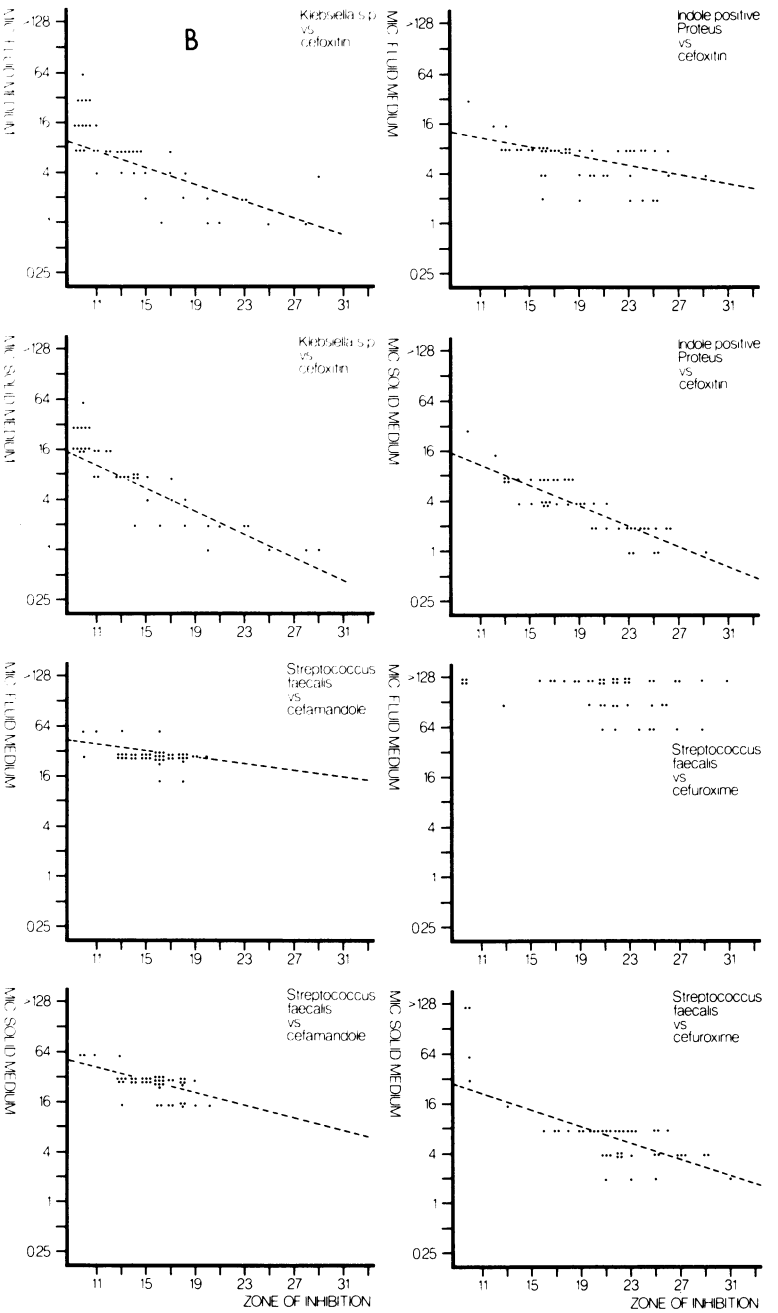

FIG. 2B

ing, its growth was not affected by cefamandole. It is of interest that cefamandole could be demonstrated in the tissue chambers in the beginning of the treatment period but not after 2 days of treatment, probably indicating that the strain produced an inducible β-lactamase. Strain P56 did not produce β-lactamase, and the cefamandole concentration in the tissue chambers exceeded by about 10-fold that required to inhibit the growth of the strain both in broth and on agar. Despite this, the reduction in viable count was slow in the tissue chambers.

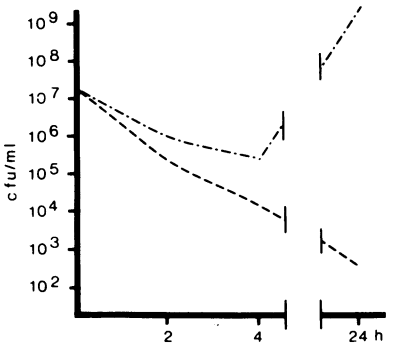

FIG. 3. *Viable counts of two indole-positive Proteus strains in PDM broth containing 25 μg of cefamandole per ml. P56 (- - -); P68 (- - - -).*

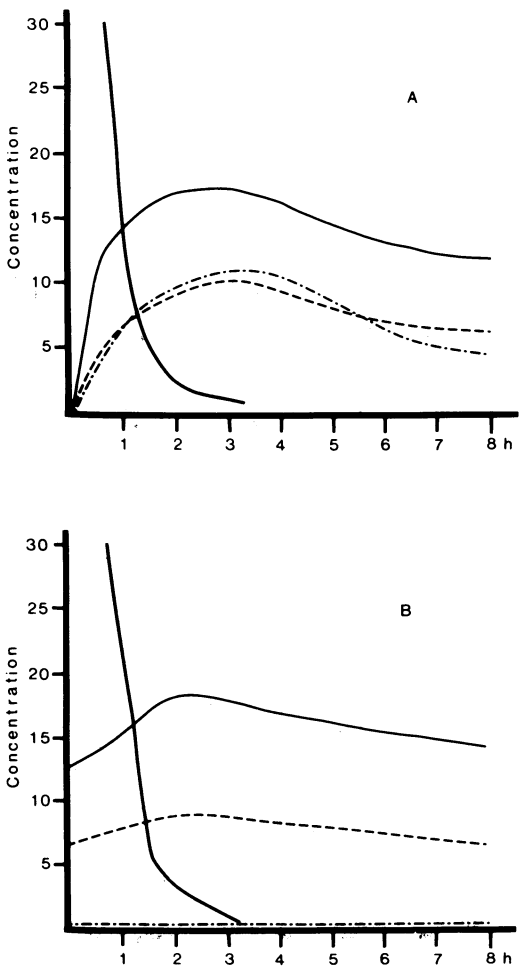

FIG. 4. *Concentration in micrograms per milliliter of cefamandole in serum and in tissue cage fluid. (A) After the first intravenous injection (50 mg/kg). (B) After intramuscular injection on day 3 (50 mg/kg). Concentrations in serum (——), in uninfected control cage (——), and in cages infected with P56 (- -) and P68 (- - -).*

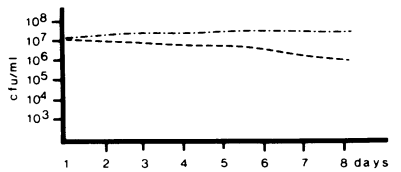

FIG. 5. *Viable counts in tissue cages infected with P56 (- -) and P68 (- - -) during treatment with cefamandole.*

TABLE 1. *Correlation between discrepant agar-broth cefamandole MICs and beta-lactamase production of indole-positive Proteus*

MIC discrepancy (fold)	No. of strains	
	Beta-lactamase producers	Beta-lactamase nonproducers
0	0	3
2	3	5
4	10	1
8	6	6
16	4	3
32	3	2
64	2	

More work is needed to elucidate the clinical importance of the observed differences in agar and broth MICs. At present, it should be observed that the size of the zone of inhibition around an antibiotic-containing disk seems to give an overoptimistic impression of the efficacy of certain cephalosporin antibiotics when tested against certain bacterial species.

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