COMPARATIVE BACTERICIDAL ACTIVITIES OF BETA-LACTAM ANTIBIOTICS DETERMINED IN AGAR AND BROTH MEDIA

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Comparative bactericidal activities were determined utilizing a relatively large number of test strains, in both agar and broth media, with special reference to the time of exposure of the bacteria to certain β-lactam antibiotics. It was apparent that the activities increase with time. The concentrations producing a 99.9% kill with cephalothin for *Escherichia coli, Klebsiella* sp., and carbenicillin for *Pseudomonas aeruginosa* were higher in broth than in agar. In contrast, those of benzylpenicillin for α-streptococcus (non-enterococcal) were higher in agar than in broth. If the bactericidal concentrations with 3-hour or 6-hour exposure to antibiotics were used as the criterion, these concentrations of carbenicillin for *P. aeruginosa*, and benzylpenicillin for α-streptococcus were, in particular, unusually high compared with the conventionally determined bacteriostatic concentrations (MICs).

Determination of bactericidal activity is widely accepted to be more important than that of bacteriostatic activity in the treatment of patients with infective endocarditis and presumably in patients with seriously impaired host defence mechanisms. The present paper describes minimal lethal concentrations (MLCs), defined as the concentrations producing a 99.9% kill[1,2], together with bacteriostatic concentrations of β-lactam antibiotics for α-streptococcus which is the major blood isolate from infective endocarditis[3,4], and also *Escherichia coli, Klebsiella* sp. and *Pseudomonas aeruginosa* which are the most frequently encountered infective pathogens in debilitated patients[5~8]. In many of the studies already reported, bactericidal activity is expressed in terms of the results obtained after overnight exposure of bacteria to antimicrobial agents[2,9,10]. Such a long period of continuous exposure of microorganisms to the antimicrobic does not generally occur in clinical practice. Most drugs are administered orally or parenterally, achieving in serum or at the focus of infection antimicrobic concentrations which are higher than the *in vitro* bactericidal concentrations for a few hours at the most. Based on this reason, we now report on *in vitro* bactericidal activities after varying periods of exposure of bacteria to β-lactams in agar and broth media.

Materials and Methods

Bacterial strains

Recent clinical isolates of *E. coli*, *Klebsiella* sp., *P. aeruginosa*, and α- and β-streptococcus (non-enterococcal) were used.

Media

Heart infusion broth (Difco Laboratories) and heart infusion agar (Difco) were used. Defibrinated horse blood (5%) was supplemented to the agar for the study of α- and β-streptococcus.

Antibiotics

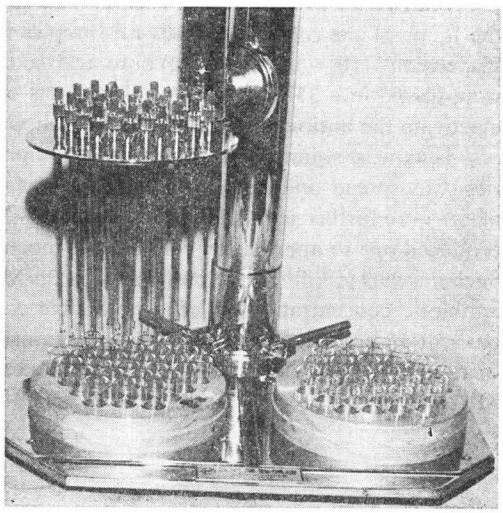
Laboratory standards of benzylpenicillin, carbenicillin and cephalothin were supplied by various pharmaceutical companies.

Determination of MLCs in broth

Stainless steel plates were used to hold a total of 27 small test tubes (12 mm in outer diameter and 20 mm in height) (Fig. 1). Each tube was filled with 1 ml of broth medium containing doubling dilutions of antibiotic (tubes on a single container plate contained a single drug concentration). A control plate with tubes containing 1 ml of antibiotic-free broth medium was also prepared. These tubes were inoculated with overnight cultures of test strains, the viable counts of which were also made by the method of MILES and MISRA[11], so as to contain 10^6 or 10^7 colony-forming units (CFUs) per ml of medium (referred to as "standardized suspension"). Inoculation was carried out using a replicator similar to the one described by STEERS *et al.*[12] For the Gram-negative strains, which yielded 10^9 or 10^{10} CFUs/ml in overnight cultures, 0.001-ml loops were used (Fig. 1). For the α- and β-streptococcus where the counts in overnight cultures were 10^7 or 10^8 CFUs/ml (Table 1) 0.1-ml pipettes were used.

Fig. 1. An apparatus used to transfer CFUs.

Two stainless steel plates each holding 27 small test tubes containing broth medium (with or without antibiotic) are placed on either side of the base of an inocula replicator. A tube-to-tube transfer of CFUs was performed by left-to-right shift of the 27 loops (0.001-ml) seen above the container plate on the left. In a tube-to-plate transfer the container plate at right was replaced by an agar plate.



The tubes were then incubated at 35°C. After 3, 6, 18 and 42 hours of incubation, a gentle vortex mix on each tube was performed to achieve a uniform suspension and using the loops of the inocula replicator 0.001-ml samples were subcultured onto β-lactamase-treated agar plates (detailed below) to determine the viable count. Assuming that there had been no change in the viable count in broth medium (with and without antibiotic) during the incubation at 35°C, the number of CFUs subcultured on agar surface with 0.001-ml loops should be 10^3 or 10^4 per inoculum. Therefore, on the plates where inocula were subcultured from the broth with a 99.9% kill antibiotic activity, one to approximately ten colonies should be seen after incubating these plates at 35°C for 18~20 hours[1]. Comparing the number of CFUs in the original culture and the colonies formed on the plates, the minimal antibiotic concentrations producing a 99.9% kill were determined and designated as 3h-MLC, 6h-MLC, 18h-MLC and 42h-MLC according to the period of incubation before samples were taken for subculture.

The minimal antibiotic concentration yielding no turbidity of the broth culture at 18 hours of

Table 1. Number of CFUs in overnight culture and the colonies formed on plates producing a 99.9% kill.

Organism	No. of strains	CFUs/ml in overnight culture	CFUs/ml in "standardized suspension"*	No. of colonies formed on plates producing a 99.9% kill
E. coli	27	$1.0\times10^9\sim5.2\times10^9$	$1.0\times10^6\sim5.2\times10^6$	1.0~5.2
Klebsiella sp.	19	$1.4\times10^9\sim5.7\times10^9$	$1.4\times10^6\sim5.7\times10^6$	1.4~5.7
P. aeruginosa	26	$1.4\times10^9\sim1.2\times10^{10}$	$1.4\times10^6\sim1.2\times10^7$	1.4~12
α- and β-Streptococcus	18	$1.0\times10^7\sim4.7\times10^9$	$1.0\times10^6\sim4.7\times10^7$	1.0~47

* Each "standardized suspension" (see text) was transferred onto agar plates by means of a 0.001-ml calibrated loop.

incubation was designated as minimal inhibitory concentration (MIC).

Preparation of β-lactamase-treated agar plates for detecting viable CFUs

Antibiotic-free agar plates, of which the entire surface was uniformly treated with 0.1-ml β-lactamase solution per plate using a perfume atomizer, were prepared for subculturing the broth medium to detect surviving CFUs. Using these plates it was assumed that the enzyme would inactivate the antibiotic activity of the subculture spotted on the agar surface thus allowing the surviving CFUs to regrow.

Determination of MLCs in agar

Four series of agar plates, each containing doubling dilution of antibiotic were prepared. Using the loops of the replicator, 0.001-ml samples were transferred from the antibiotic-free "standardized suspension" ($10^6 \sim 10^7$ CFUs/ml) onto agar so as to deliver $10^3 \sim 10^4$ CFUs per inoculum. These plates were placed in a 35°C incubator. The 4 sets of plates were then treated with β-lactamase solution to inactivate the antibiotic at 3, 6, 18 and 42 hours of incubation, respectively. For this purpose, 0.1 ml of β-lactamase solution (crude enzyme solution of cephalosporinase or Penase of the Difco Laboratories) was spread uniformly over the agar surface of each plate using a perfume atomizer[2,13]. These plates were further incubated at 35°C for 18 ~ 20 hours. The minimal antibiotic concentrations which produced one to approx. ten colonies per inoculum on the plates, indicative of the concentrations producing a 99.9% kill[2], were designated as 3h-MLC, 6h-MLC, 18h-MLC and 42h-MLC. The minimal antibiotic concentration yielding no visible colonies at 18-hour incubation without β-lactamase treatment was also determined and designated as MIC.

Results

Comparative MLC values

Fig. 2 shows cephalothin MLCs for 27 strains of *E. coli*. With cephalothin a level as high as 50 μg/ml can sometimes be achieved in serum, and in agar this concentration was lethal to 11 and 21 out of the 27 strains when 3h- and 6h-MLCs, respectively, were used as the criterion. In broth this concentration was lethal to 4 and 18 strains when assessed with 3h- and 6h-MLCs, respectively. Conventional MICs roughly agreed with 18h- and 42h-MLCs determined in agar and in broth and also with 6h-MLC in broth. The values were usually several-fold higher in broth than in agar.

The results with cephalothin and 19 strains of *Klebsiella* sp. are shown in Fig. 3. The 3h- and 6h-MLCs were higher than the 18h- and 42h-MLCs. The latter values were similar to the MICs in the two different media. MICs and MLCs were also higher in broth than in agar.

Fig. 4 shows the results obtained with 26

Fig. 2. MLCs of cephalothin for 27 strains of *E. coli*.

Results show cumulative numbers of strains killed by increasing antibiotic concentrations. The 3h-, 6h-, 18h- and 42h-MLCs are the concentrations which produced lethal effect (MLC) with the indicated time of exposure to the drug. Conventional MICs are also shown.

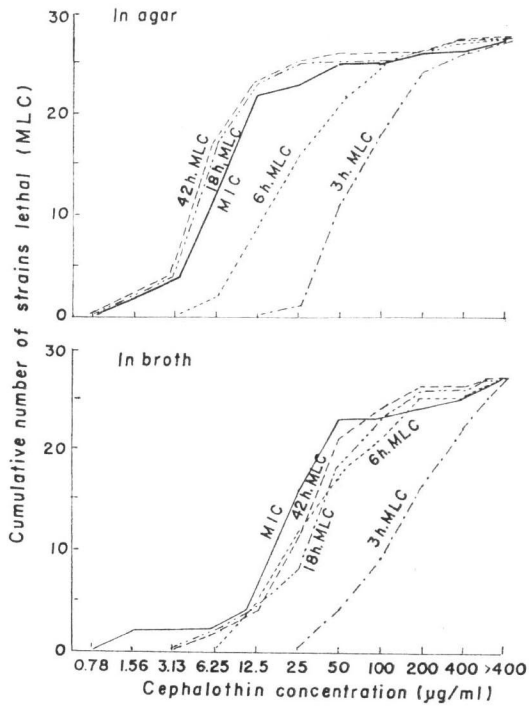


Fig. 3. MLCs of cephalothin for 19 strains of *Klebsiella* sp.

Abbreviations and symbols are the same as in Fig. 2.

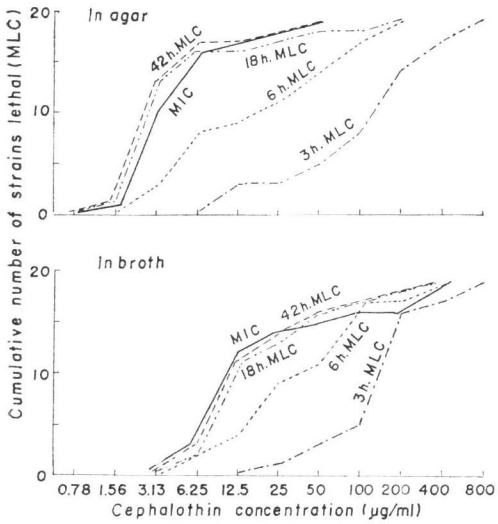


Fig. 4. MLCs of carbenicillin for 26 strains of *P. aeruginosa*.

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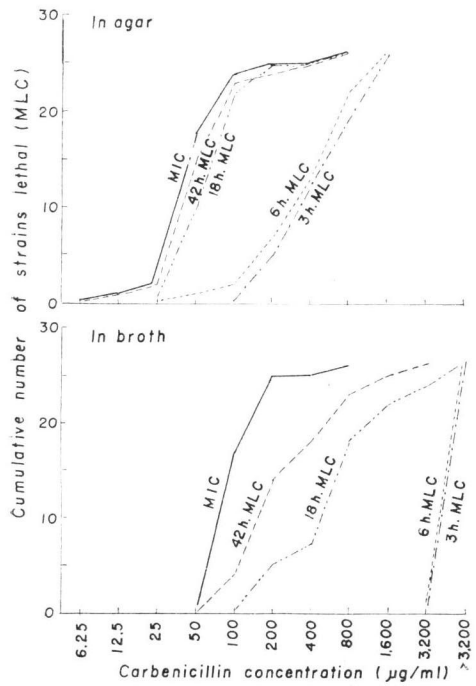
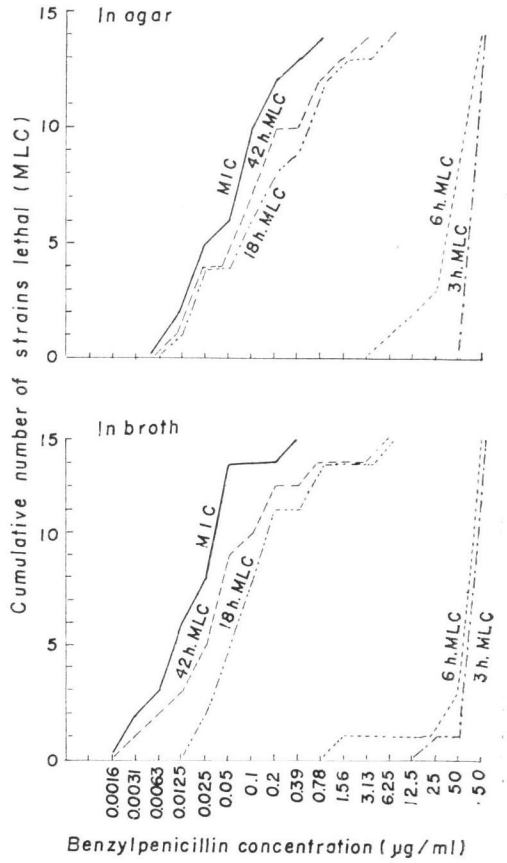


strains of *P. aeruginosa* and carbenicillin. Noteworthy is the unusually high MLCs in broth, in particular the 3h- and 6h-MLCs which exceeded 3,200 μg/ml indicating that there had been no strain yielding a 99.9% decrease of viable count after exposure to this antibiotic at a concentration of 3,200 μg/ml. Broth medium 18h- and 42h-MLCs were also higher than the MIC values.

With benzylpenicillin, fourteen strains of α-streptococcus showed 3h- and 6h-MLCs which were considerably higher than the MICs in agar and in broth (Fig. 5). In contrast to the Gram-negative strains, MIC values were higher in agar than in broth.

Fig. 5. MLCs of benzylpenicillin for 14 strains of α-streptococcus.

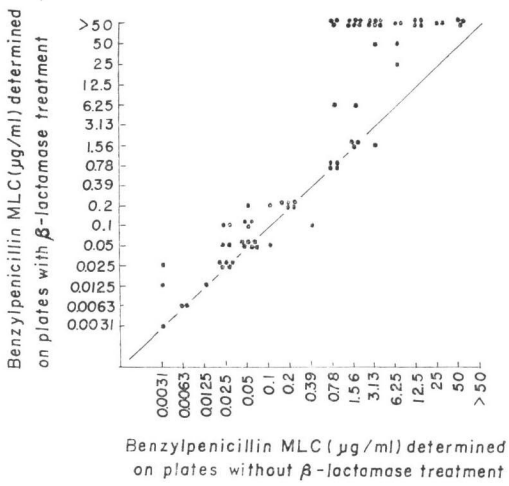
Abbreviations and symbols are the same as in Fig. 2.



Broth Medium MLC Results Obtained with β-Lactamase-treated and -Untreated Agar Plates

The MLCs (including 3h-, 6h-, 18h- and 42h-MLCs) determined in broth utilizing α- and β-streptococcus (14 and 4 strains, respectively) with benzylpenicillin varied when the agar plates for detecting viable CFUs were treated or not treated with β-lactamase solution (Fig. 6). A number of strains, for which the MLC values obtained on β-lactamase-treated plates were in excess of 50 μg/ml, showed MLC values ranging from 0.78 to 25 μg/ml when determined on plates without β-lactamase treatment. A good agreement was seen between β-lactamase and non-β-lactamase plates when the benzylpenicillin concentration was below 0.39 μg/ml.

Fig. 6. Comparison of MLC results determined on plates with and without β-lactamase treatment.
α-Streptococcus (14 strains) and β-streptococcus (4 strains) with benzylpenicillin were used in this study.



Similar results were obtained with Gram-negative bacteria and carbenicillin or cephalothin (not shown). These results show the importance of inactivating the antibiotic when carrying out determinations of viable count.

Discussion

It has been fully described elsewhere using regression curves for individual microbial strains in broth medium, that the viable count of common bacteria decreases with time in the presence of high concentrations of bactericidal drugs[14,15]. Our present paper has detailed a method for determining quantitative bactericidal activities along with bacteriostatic ones, using a relatively large number of test strains, both in agar and broth media, with special reference to the time of exposure to drugs. The study confirmed that the bactericidal activities increase with time. Bacteriostatic and bactericidal activities were lower in broth than in agar for the Gram-negative species. The comparative bacteriostatic data were in agreement with those of other investigators[16]. In contrast to the Gram-negative organisms the bacteriostatic and bactericidal concentrations were higher in agar than in broth with the α-streptococcus. The addition of horse blood to agar but not to broth, and the clump-forming tendency of the α-streptococcus seen in the heart infusion broth may be responsible for these results. The bactericidal effect over comparatively short periods of time, namely 3 hours and 6 hours, was also investigated. The bactericidal concentrations for *P. aeruginosa* and α-streptococcus were, in particular, markedly high compared with the bacteriostatic levels when they were assessed in terms of these short periods of exposure. These data suggest that it would be difficult to treat infections with such strains by administering these antibiotics in a way which produces in serum or infected foci only a brief time span of antimicrobic concentration exceeding the *in vitro* bactericidal levels.

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