

STUDIES ON BACTERICIDAL ACTIVITIES OF β -LACTAM ANTIBIOTICS ON AGAR PLATES: THE CORRELATION WITH THE ANTIBACTERIAL ACTIVITIES DETERMINED BY THE CONVENTIONAL METHODS

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A simplified method to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of β -lactam antibiotics on agar plates is described. MIC values were determined on agar plates for benzylpenicillin, methicillin and cephalothin using *Staphylococcus aureus* and *Klebsiella pneumoniae*. A β -lactamase solution was then sprayed onto the plates to inactivate the drug(s). After further incubation at 37°C overnight, the minimal concentration at which no test bacteria were visible on the plates was defined as MBC. Both MIC and MBC values decreased with decreased inoculum size. The two values were almost coincidental when high dilutions were used as the inocula. These values were compared with those obtained by the conventional broth dilution method. In this study, MIC as well as MBC values determined by the simplified method were generally smaller than the values determined by the broth dilution technique.

Antibiotic susceptibility tests determining minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) are often employed in the treatment of bacterial infections. MBC values are usually determined by subculturing the broth of clear tubes onto agar plates following the broth dilution MIC determination; however the method is time-consuming.

A simplified method to determine MIC and MBC values of β -lactam antibiotics on agar plates is described.

Materials and Methods

Bacteria. All strains were isolated recently from clinical specimens at the Keio University Hospital, Tokyo. They were identified as 6 strains of *Staphylococcus aureus* and 5 strains of *Klebsiella pneumoniae*. All of the *S. aureus* were penicillinase producers, as determined by the modified GOTS method¹⁾.

Antibiotics. Commercial preparations of benzylpenicillin (Takeda Chemical Co., Ltd., Osaka), methicillin (Banyu Pharmaceutical Co., Ltd., Tokyo) and cephalothin (Shionogi Pharmaceutical Co., Ltd., Osaka) were employed. A phosphate buffer solution (pH adjusted to 7.2) was used for the dilution of antibiotics and specimens throughout the experiments.

Media. Heart infusion agar (Difco) and heart infusion broth (Difco) were used.

β -Lactamases. A commercial preparation of penicillinase (Bacto-Penase 50,000 units/ml; Difco) was used to inactivate the penicillins. Crude enzyme solution for inactivating cephalothin was obtained by filtering the broth culture of a strain of *Enterobacter cloacae*, known to be a potent β -lactamase (cephalosporinase) producer. One ml of the filtrate was capable of inactivating 300,000 μ g of cephalothin in liquid medium²⁾.

Determination of MIC and MBC values on agar plates. Serial twofold dilutions of each β -lactam antibiotic were prepared in heart infusion agar in 90-mm petri dishes. The inocula were undiluted and 10^{-2} , 10^{-4} and 10^{-6} dilutions (expressed as 1:1, 1:100, 1:10,000 and 1:1,000,000 respectively in Figs. 1, 2 and 3) of overnight cultures. The undiluted cultures contained $10^8 \sim 10^9$ viable cells per ml each.

Each inoculum was removed by the use of a 0.001 ml calibrated loop. After determination of the MIC values according to the conventional manner, the β -lactamase solution was sprayed onto the agar plates with a perfume atomizer and the plates incubated overnight at 37°C. The amount of the enzyme solution used was *ca.* 0.1 ml per plate. In this method, MBC values were defined as the minimal concentrations of the drug(s) at which no visible growth of test bacteria was observed on the plates following incubation.

Determination of MIC and MBC values in broth. A series of test tubes each containing 2.5 ml of heart infusion broth with twofold dilutions of the β -lactam antibiotic were prepared. The inocula were undiluted and 10^{-2} and 10^{-4} dilutions of overnight cultures; 0.025 ml of each of these inocula was introduced into each of the 2.5-ml test tubes, resulting in final dilutions of *ca.* 1: 100, 1: 10,000 and 1: 1,000,000, respectively, of the original culture. Each of these dilutions was compared with each of the 10^{-2} , 10^{-4} and 10^{-6} dilutions in the agar dilution method. After incubation at 37°C overnight, the MIC values were determined, followed by subculturing the broth onto agar plates to determine MBC values according to the conventional manner.

Demonstration of inactivation of cephalothin by the application of β -lactamase (Fig. 4). An agar plate containing 400 μ g of cephalothin per ml of medium was inoculated at opposite sides of the plate with an undiluted culture of *K. pneumoniae*. The plate was incubated at 37°C overnight at which time no bacterial growth was observed. The β -lactamase solution was sprayed onto the right hand-side of the plate and the plate incubated again at 37°C overnight.

Results

MIC and MBC Values

The values obtained by the two methods were compared at the several inoculum levels. As presented in Fig. 1, the MIC and MBC values of benzylpenicillin for *S. aureus* decreased with diminution of the inoculum size both in agar and in broth. The values obtained by the agar dilution method (Fig. 1, above) were smaller than those obtained by the broth dilution method (Fig. 1, below). In agar, at the inoculum size of 1: 100, the range of MIC values was 1.56~25 μ g/ml, while that for the MBC values was 1.56~800 μ g/ml; in contrast the MIC and MBC values were almost equivalent when the greatest dilutions (1: 10,000 and 1: 1,000,000) were used. By the broth dilution method (Fig. 1, below), the range of MIC was 400~1,600 μ g/ml or more, and 3,200 μ g/ml or more for the MBC values when a 1: 100 dilution was used. These values also decreased as the dilution of inoculum increased.

A similar study was performed with methicillin and *S. aureus* (Fig. 2). By the agar dilution method, the range of MIC and MBC values decreased with increased dilution of the inoculum. The two values essentially coincided when 1: 10,000 and 1: 1,000,000 dilutions were used. By the broth dilution method, both the MIC and MBC values were greater than those obtained by the agar dilution method.

The experiment was also carried out with cephalothin and *K. pneumoniae* (Fig. 3). At the inoculum size of 1: 100, the MIC values obtained by the broth dilution method were twofold higher than those obtained by the agar dilution method; whereas the MIC values of the two methods were almost the same when the greatest dilutions (1: 10,000 and 1: 1,000,000) were used.

Demonstration of Inactivation of Cephalothin by the Application of β -Lactamase

As seen at the right hand-side of the plate in Fig. 4, growth of *K. pneumoniae* appeared where bacterial cultures had been inoculated with the addition of β -lactamase, whereas no growth appeared at the left hand-side where the enzyme had not been applied. The bacterial growth demonstrates that cephalothin has lost its potency on the plate due to the action of β -lactamase.

A similar control study was carried out with each antibiotic to confirm drug inactivation as part of

Fig. 1. Effect of the method of determining MIC and MBC values as related to inoculum size.

MIC and MBC values were determined by the agar dilution method (above) and by the broth dilution method (below). Six strains of *Staphylococcus aureus* were used with benzylpenicillin.

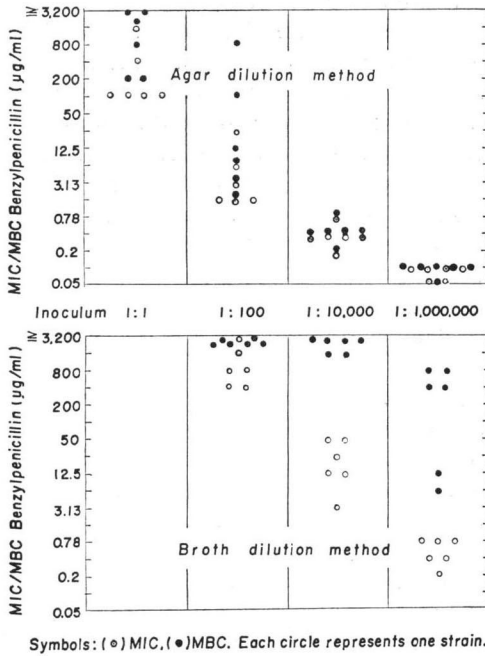


Fig. 2. Effect of the method of determining MIC and MBC values as related to inoculum size.

MIC and MBC values were determined by the agar dilution method (above) and by the broth dilution method (below). Five strains of *Staphylococcus aureus* were used with methicillin.

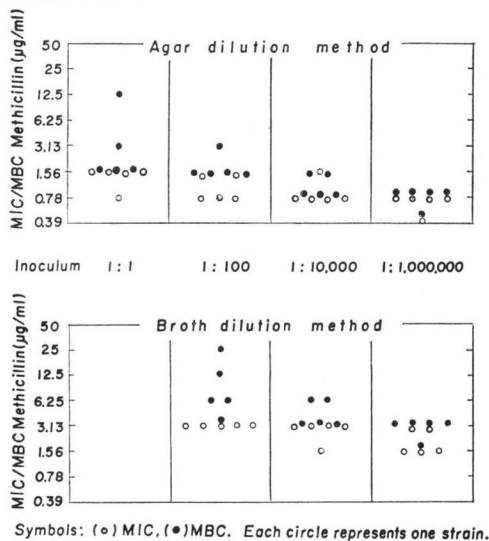


Fig. 3. Effect of the method of determining MIC and MBC values as related to inoculum size.

MIC and MBC values were determined by the agar dilution method (above) and by the broth dilution method (below). Five strains of *Klebsiella pneumoniae* were used with cephalothin.

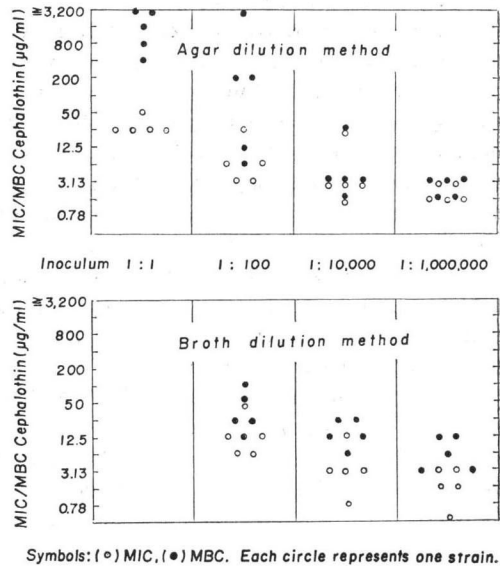
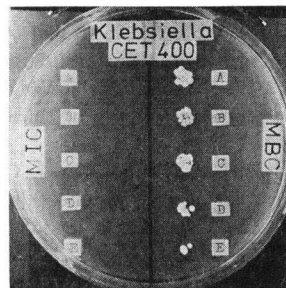


Fig. 4. Inactivation of cephalothin by the application of β -lactamase.

The agar plate contained 400 µg/ml of cephalothin. An undiluted culture of *Klebsiella pneumoniae* was inoculated at opposite sides of the plate, as indicated by positions A to E. The plate was incubated at 37°C overnight. At this time, no bacterial growth was observed at any point of inoculation as seen on the left hand-side of the plate ("MIC" side). β -Lactamase solution was then sprayed onto the right hand-side of the plate. After further incubation at 37°C overnight, bacterial growth appeared in the area treated with the enzyme ("MBC" side).



each experiment.

Discussion

In this paper, bactericidal activities of β -lactam antibiotics were studied by using a novel method which had been developed in the author's laboratory. The activities were also compared with the MIC and MBC values determined by the conventional agar and broth dilution methods.

In the present study, the cell concentration in the inoculum was taken into consideration in the comparison of the values obtained by the two different methods. In the broth dilution method, an aliquot of the undiluted culture was added to 100 aliquots of the broth resulting in *ca.* 1:100 dilution. This was regarded as corresponding with the 10^{-2} dilution in the agar dilution method. In the same way, an aliquot of the 10^{-2} and 10^{-4} cultures added to 100 aliquots of broth in the broth dilution method was regarded as corresponding with 10^{-4} and 10^{-6} dilutions in the agar dilution method respectively. The comparison was almost in correspondence with that of JACKSON *et al.*³⁾. It is generally agreed that the MIC values of β -lactam antibiotics depend largely upon inoculum size^{4,5,6)}. The same correlation was also observed between MBC values and inoculum size. It is generally accepted that MIC values tend to be higher in broth than in agar⁴⁾. In the present study, MBC values obtained by the broth dilution method were also higher than those obtained by the agar dilution method. However, its significance in clinical practice is controversial.

In the presented agar plate method, MBC values can be determined when complete inactivation of the antibiotic occurs on the plates by the β -lactamase treatment. Therefore, the values also depend largely upon stability of the antibiotic to the enzyme and activity of the β -lactamase employed. Hence, the possibility remains that in some cases the presence of small amounts of residual antibiotic on the agar surface after the enzymatic treatment will influence the MBC values determined by the agar dilution technique.

The agar dilution method is a convenient tool to assess bacteriostatic activities of antibiotics against a number of strains. In some special clinical situations, however, bactericidal activities are considered to be more important than bacteriostatic activities. By the use of the presented agar plate method, bactericidal activities can also be assessed against a number of bacterial strains. The author recommends this method as a simplified procedure to determine MBC values as well as MIC values in the laboratory.

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