Bacteriostatic and Bacterial Activities of Selected Beta-Lactam Antibiotics Studied on Agar Plates

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A novel and time-saving method for assessing bactericidal activities of β-lactam antibiotics on agar plates is described. Minimal inhibitory concentrations (MICs) were determined by the agar dilution method. A potent β-lactamase solution was sprayed onto the plates to inactivate the antibiotic. After further incubation at 37°C overnight, the minimal concentration at which no visible growth occurred on the plates was defined as minimal bactericidal concentration (MBC). With undiluted culture as the inoculum, strains of Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae showed a marked increase in MBC values compared with the values of MIC. There was a marked decrease in both the MICs and MBCs with diminution of inoculum size. The two concentrations were almost the same when the inoculum size was decreased to a 10⁻⁴ dilution. In contrast, MIC and MBC for enterococci showed no marked decrease with decrease in inoculum size. Although the present study was performed with β-lactamase-unstable penicillins and cephalosporins, the method can be applicable with any β-lactam antibiotic if optimal and potent enzymes are available.

In some special clinical situations, minimal bactericidal concentrations (MBCs) should be determined rather than minimal inhibitory concentrations (MICs) of the antibiotic to be used. At present, MBC can be determined by a loop transfer method following the broth dilution MIC determination; however, the whole procedure is time-consuming.

In the present paper, a simple method is described to assess bactericidal activities of selected β-lactam antibiotics on agar plates.

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MATERIALS AND METHODS

Bacterial strains. All clinical strains were isolated recently at the Keio University Hospital, Tokyo. They were identified as Staphylococcus aureus (18 strains), enterococcus (20 strains), Escherichia coli (18 strains), and Klebsiella pneumoniae (25 strains). The standard strain of S. aureus 209P was also used.

Antibiotics. Each of the laboratory standards was supplied by the pharmaceutical company listed in parentheses after the antibiotic: benzylpenicillin (Takeda Chemical Co., Ltd., Osaka), ampicillin (Fujisawa Pharmaceutical Co., Ltd., Osaka), amoxicillin (Fujisawa Pharmaceutical Co., Ltd., Osaka), cephaloridine (Shionogi Pharmaceutical Co., Ltd., Osaka), cephalothin (Shionogi Pharmaceutical Co., Ltd., Osaka), cefazolin (Fujisawa Pharmaceutical Co., Ltd., Osaka), and cefazolin (Fujisawa Pharmaceutical Co., Ltd., Osaka). 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 (Penase, Difco; 50,000 U/ml) was used to inactivate the penicillins. One milliliter of the enzyme solution inactivated up to 5,000,000 μg of each benzylpenicillin, ampicillin and amoxicillin in liquid medium, as assayed by the method described elsewhere (6). A crude enzyme solution obtained by filtering the broth culture of a strain of Enterobacter cloacae, known to be a potent β-lactamase producer, was used to inactivate the cephalosporins. One milliliter of the solution was capable of inactivating 350,000 μg of cephaloridine, 300,000 μg of cephalothin or cefazolin, and 200,000 μg of cephalaxin, respectively, in liquid medium. This solution was kept at −20°C in a deep freezer until used.

Determination of MIC and MBC in agar. Heart infusion agar plates (10 ml) with serial twofold dilutions of each β-lactam antibiotic were prepared in 90-mm petri dishes. Inoculum sizes were undiluted (10⁶ to 10⁸ cells/ml), and overnight cultures were diluted to a 10⁻² dilution (10⁶ to 10⁷ cells/ml) and a 10⁻⁴ dilution (10⁴ to 10⁵ cells/ml). Each of these inocula was inoculated on the agar plates by the use of inocula-replicating apparatus and transferred by a
0.001-ml calibrated loop. These plates were incubated at 37°C overnight. The MIC was defined as the minimal antibiotic concentration that yielded complete inhibition of the bacteria on the plates. β-Lactamase solution was then sprayed uniformly onto the plates by using a perfume atomizer. The amount of the enzyme solution added was about 0.1 ml per plate. These plates were incubated again at 37°C overnight. The minimal antibiotic concentration at which no visible growth occurred on the plates after incubation was arbitrarily defined as MBC. MIC and MBC values were studied in relation to inoculum size.

Confirmative study of antibiotic inactivation on the agar plates. A control study was also carried out simultaneously using the same agar plates that were being used for the determination of MBC values. The strain most susceptible to the antibiotic, the MIC of which was being determined by the study, was selected as the control organism. A loopful (0.001 ml) of a 10⁻⁴ culture (the greatest dilution used in the study) of the control strain was additionally inoculated on the agar plates that had already undergone β-lactamase treatment. After further incubation at 37°C overnight, the plates showing visible growth of the control bacteria were considered to be optimal for determining MBC values.

Diffusion of β-lactamase into the agar layer. Five milliliters of heart infusion agar containing 1,600 μg of ampicillin per ml of medium was poured into a 90-mm petri dish. After drying, four stainless-steel cylinders (8 mm in diameter) were placed on the agar plate. Additionally, 5 ml of heart infusion agar containing 1,600 μg of ampicillin and cells of S. aureus 209P in the concentration of about 10⁷ viable cells per ml of medium were poured onto the plate. After solidification, the cylinders were removed, resulting in holes in the plate. A 0.025-ml sample of undiluted and 10-fold, 100-fold, and 1,000-fold diluted penicillinase solutions (Penase, 50,000 U/ml) was introduced in each of the holes on the plate. This plate was allowed to stand in a freezer at 4°C up to 3 h. The plate was then incubated at 37°C overnight (Fig. 1).

Demonstration of inactivation of ampicillin by the application of β-lactamase. A heart infusion agar plate containing 1,600 μg of ampicillin per ml of medium was prepared. A 0.1-ml sample of the penicillinase solution was sprayed onto the agar plate. After drying, each of the undiluted and 10⁻¹,
**RESULTS**

**MIC and MBC values.** Figure 3 represents the cumulative percentage of 18 strains of *S. aureus* inhibited (MIC) and/or killed (MBC) by benzylpenicillin, ampicillin, and amoxicillin. With undiluted culture as the inoculum, almost 50% of the strains were inhibited by 25 μg of either of these antibiotics per ml, whereas concentrations as high as 1,600 μg of these drugs per ml killed only 40 to 50% of the strains. The MIC and MBC values markedly decreased in accordance with diminution of inoculum size. The two values almost agreed when the 10⁻⁴ dilution was used as the inoculum. A concentration of 1.56 μg of these drugs per ml was bacteriostatic or bactericidal for 80 to 90% of these strains. With this dilution, 12.5 μg of these drugs per ml achieved bacteriostatic and bactericidal effect for all the strains tested.

Figure 4 depicts the result obtained with enterococci and the three penicillin antibiotics. With undiluted culture as the inoculum, 6.25 μg of benzylpenicillin, 3.13 μg of ampicillin, or 0.78 μg of amoxicillin per ml each completely inhibited the growth of the test strains, whereas an excess of 1,600 μg of each of these drugs per ml was needed to kill all of the strains tested. In contrast to the *S. aureus*, the inoculum effect of enterococci on MIC and MBC values was smaller. At the 10⁻⁴ dilution, MIC values of the three penicillins decreased about twofold more than those obtained with the undiluted culture. Likewise, MBC values did not show a marked decrease with decrease in inoculum size.

The result with *E. coli* and the two penicillins and cephalosporins is presented in Fig. 5. With undiluted culture as the inoculum, ceftazolin showed the most potent bacteriostatic activity to all of the *E. coli* strains. Cephalothin was less potent bacteriostatically than ampicillin and amoxicillin for almost one-half of the
strains, whereas the remaining half were resistant to 1,600 μg of ampicillin and amoxicillin per ml. Such resistant strains also showed high MIC and MBC values at the inoculum size of $10^{-4}$ dilution.

Figure 6 represents the result using 25 strains of K. pneumoniae with cephaloxin, cephaloridine, cephalothin, and cefazolin. With undiluted culture, 25 μg of any of these cephalosporin antibiotics per ml was bacteriostatic for 70 to 85% of the Klebsiella stains. In contrast, 1,600 μg of these drugs per ml was bactericidal for less than 50% of the strains of K. pneumoniae. A marked decrease in MIC and

![Diagram](http://aac.asm.org)
MBC values was obtained in accordance with the decrease in inoculum size. At the 10⁻⁴ dilution, as high as 6.25 µg of any of these four cephalosporins per ml showed the activity of inhibiting and/or killing to about 80 to 90% of the strains. With this light inoculum, the concentration of 12.5 µg of cephalexin or cefazolin per ml and 25 µg of cephalothin or cephaloridine per ml was enough to inhibit or kill all the strains of K. pneumoniae used in the study.

Diffusion of β-lactamase into the agar layer. Growth of S. aureus 209P became visible in and on the agar plate to a significant extent surrounding the holes that contained high concentrations of penicillinase solution (Fig. 1). The growth demonstrates the inactivation of ampicillin by the potent penicillinase used. The result suggests that the β-lactam antibiotic significantly loses its activity in the presence of a potent enzyme.

Demonstration of inactivation of ampicillin on an agar plate. Cells of S. aureus 209P formed colonies on the agar plate (Fig. 2). The colonies indicated by the position "-4" originated from the inoculum of a 10⁻⁴ dilution (10 to 10⁶ viable cells). This inoculum was the most susceptible to ampicillin among the variously diluted inocula used to study inoculum effect. Such growth demonstrates that ampicillin has significantly lost its potency on the agar surface due to the action of β-lactamase.

DISCUSSION

In this paper we report on comparative studies on bacteriostatic and bactericidal activities of β-lactam antibiotics performed by use of a novel method which had been developed in our laboratory.

The MICs of selected β-lactam antibiotics for several common bacteria were similar to those from other studies. Inoculum effect on MICs was also studied. It is generally accepted that inoculum has a considerable effect on MIC with the β-lactam antibiotics (3, 4). In our study, a marked inoculum effect on MICs was also demonstrated with S. aureus, E. coli, and K. pneumoniae. Here, almost half the strains of E. coli were highly resistant to ampicillin and amoxicillin, which showed no obvious inoculum effect within the range of drug concentrations studied. Presumably, this high drug resistance may be attributable to the intrinsic resistance of the bacteria. With the enterococci, the inoculum effect was smaller.

The MBCs measured were high when the inoculum size was large, whereas these values also decreased in accordance with the decrease in inoculum size. At the 10⁻⁴ dilution, MICs and MBCs for S. aureus, E. coli, and K. pneumoniae were almost identical. The MBCs for gram-negative bacteria reported from other institutions were almost the same or slightly higher than the values obtained in the present study (2, 8). A preliminary report from our laboratory with a small number of bacterial strains indicated that the MBC with a light inoculum was almost equal or larger in broth than in agar (5). A discrepancy was observed with the enterococci, however, between the MBCs obtained by the two different methods. With a light inoculum, the MBC for enterococcus strains obtained by the conventional procedure (1, 7) was considerably lower than that obtained by the agar plate method used here.

The β-lactamase solution diffused into the
FIG. 5. Bacteriostatic (MIC) and/or bactericidal activities (MBC) of two penicillins and cephalosporins against 18 strains of E. coli. Symbols: ×, ampicillin; V, amoxicillin; O, cephalothin; ○, cefazolin; ----, MIC; -----, MBC.

agar layer that produces inactivation of high concentrations of the β-lactam antibiotics adjacent to the enzyme solution. The result indicates that the antibiotic inactivation occurs on the agar surface by the β-lactamase sprayed onto the agar plate. The growth of the ampicillin-susceptible strain on the β-lactamase-treated agar plate also demonstrated that the drug concentration on the agar plate is almost negligible (Fig. 2). However, the possibility still remains that, in some instances, the small amounts of antibiotic activity remaining on the agar surface will influence the MBC values obtained by the method presented. Hence, the
use of the 10⁻⁴ dilution of the most susceptible strain in the experiment, as the indicator, may tentatively be acceptable in the method for determining MBC values.

If one wants to determine quantitative bactericidal activities of the antibiotics, i.e., the concentrations producing a 99.9% kill (arbitrarily defined as minimal lethal concentration) by the agar plate method, a 10⁻² dilution of overnight cultures (each containing 10⁶ to 10⁸ cells per ml) plated by a 0.001-ml calibrated loop (10⁸ to 10⁹ inoculated cells) is recommended. The minimal lethal concentrations could be determined as the lowest concentrations that yield no more than a few colonies at the time of MBC determination. In some instances, however, the inoculum size could be replaced by a 10⁻³ dilution with a 0.01-ml calibrated loop which also produces 10³ to 10⁴ viable cells at the time of inoculation.

Although the present study was performed only with β-lactamase-unstable penicillins and cephalosporins, the method can be applicable to any antibiotic where optimal and potent enzymes are available. The method presented is simple and time-saving for assessing bacteriostatic and bactericidal activities of β-lactam antibiotics against a number of bacterial strains. The method can be useful in the comparison of antibacterial activities of β-lactam antibiotics in the laboratory.

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LITERATURE CITED