NOTES

DETECTION OF β-LACTAMASE PRODUCTION BY GRAM-NEGATIVE BACTERIA

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It has been well documented that numbers of gram-negative bacteria produce β-lactamases which play a major role in the inactivation of penicillins and cephalosporins. In this paper, β-lactamase activity was studied by using recent clinical isolates of gram-negative bacteria obtained from patients at the Keio University Hospital, Tokyo.

Test strains were grown in heart infusion broth (Difco) at 37°C without shaking and harvested at different time intervals. These cultures were sterilized with membrane filters (pore size 0.45 μ). Different dilutions (1/5, 1/50 and 1/500) of these filtrates were mixed with different concentrations of antibiotic solutions (20, 200 and 2,000 μg/ml) respectively in equal amounts (resulting in an initial antibiotic concentration of 10, 100 or 1,000 μg/ml). The mixtures were then incubated at 37°C for 3 hours. The residual potency of the antibiotic was measured biologically. The amount of antibiotic which had been inactivated by 1 ml of the original, undiluted filtrates was considered to represent the cell-free β-lactamase activity of the bacteria. Some strains, in particular *Enterobacter* sp., showed an especially high ability to inactivate β-lactam antibiotics. One ml of these filtrates inactivated up to 300,000 μg of some β-lactam antibiotics (Figs. 1 and 4). The activity was previously reported to increase with time.[1] Many reports inform us that the β-lactamases of gram-negative bacteria are normally cell-bound and enzymes appear in the culture medium only in a small portion.[2~5,7] Therefore, the actual inactivating ability of the cell-bound β-lactamases might be far more potent than that obtained in our present study. The activity of the filtrates from 6 gram-negative bacteria was determined against 9 β-lactam antibiotics (Fig. 1). Among the penicillins, benzyl-penicillin and ampicillin were generally most readily inactivated by the filtrates and cloxacillin was the most stable; among the cephalosporins, cephaloridine was most readily inactivated and cephalexin the most stable. These data were comparable to those with cell-bound β-lactamases published elsewhere.[2~6]

As a semiquantitative, screening method for detection of β-lactamase activity, the "double disc method" has been developed (Figs. 2 and 3). Five ml of a mixture composed of 100 parts of sterilized heart infusion agar (Difco) and 1 part of an over-night culture of *Staphylococcus aureus* FDA 209P were poured into 90-mm petri dishes and dried. Each antibiotic disc (containing 30 μg of ampicillin or cephalothin respectively) was placed at the center of each of the agar plates. Discs (6.5 mm) in which test bacteria had been incorporated by dipping them in their overnight broth cultures were put on the plates at a given distance from the antibiotic discs. The distance from the center of the antibiotic disc to the distant side of the satellite discs (containing test bacteria) was 20 mm. After an incubation at 37°C for 48 hours, the growth of assay bacteria which appeared

Fig. 1. Substrate profiles of bacterial β-lactamases. The amount of β-lactam antibiotics which was inactivated by 1 ml of the filter-sterilized, 48-hour broth cultures is presented in 6 strains of gram-negative bacteria. Abbreviations: PC-G, benzyl-penicillin; AB, ampicillin; CB, carbenicillin; SB, sulbenicillin; MCI, cloxacillin; CET, cephalothin; CER, cephaloridine; CEX, cephalexin; CEZ, cefazolin.

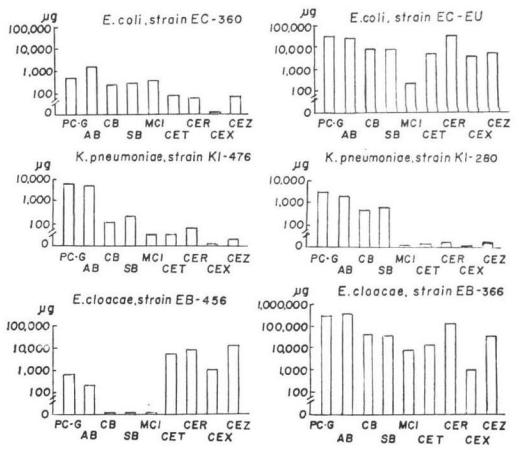


Fig. 2. The scheme of the "double disc method".
The agar plate was seeded with cells of assay bacteria. The center disc contained an antibiotic. The satellite discs of A, B, C, D and E were incorporated with overnight cultures of test bacteria. The schematic patterns appear after an incubation at 37°C for a given time.
Around each of the satellite discs proliferation zones of assay bacteria (shadowed area) materialize. They are classified into five scores from (−) to (卌) according to the degree of proliferation.

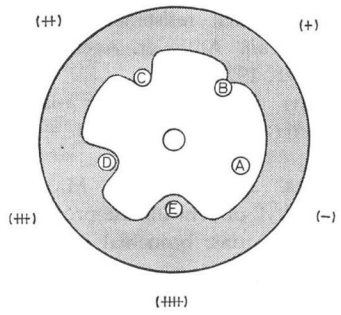


Fig. 3. An example of the "double disc method".
The agar plate was seeded with cells of *Staphylococcus aureus* FDA 209P (cell count *ca.* 10^7/ml). The central disc contained 30 μg of ampicillin. Four satellite discs were incorporated with overnight cultures of test bacteria (all of them different strains of *Enterobacter cloacae*). After an incubation at 37°C for 48 hours, proliferation of *S. aureus* FDA 209P materialized in varying degrees around each of the satellite discs.

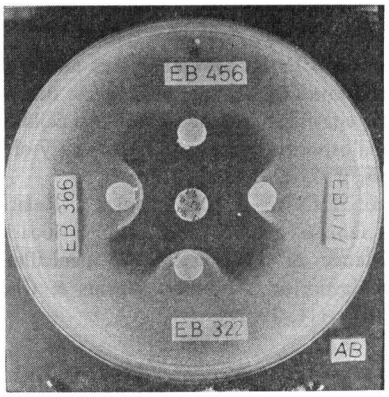
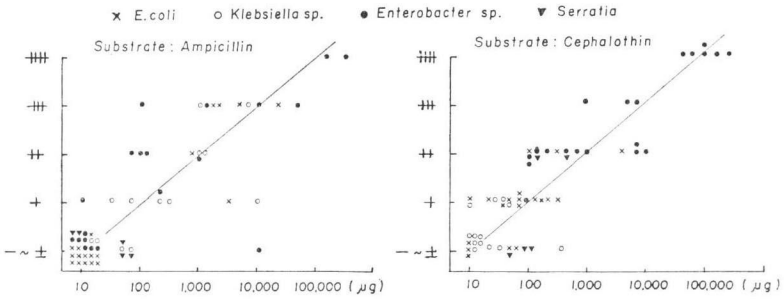


Fig. 4. Relationship of the two values, that obtained with filter-sterilized cultures and the score by the "double disc method".
β-Lactamase activity obtained with 1 ml of filter-sterilized, 48-hour cultures (abscissa) was compared with the score by the "double disc method" (ordinate). Ampicillin and cephalothin were used as the substrates.
Clinical strains of *Escherichia coli* (19 strains), *Klebsiella* sp. (13), *Enterobacter* sp. (20) and *Serratia* (5) were used in the study.



around the satellite discs was scored (Fig. 2). The score was considered to represent the β-lactamase activity of the test bacteria. Fig. 4 represents the relationship of the values with the two different methods; values measured with the filtrates and those by the "double disc method". Despite the problems of the crypticity factor and inducibility,[2~5,7] the results of the two methods with a number of gram-negative bacteria were in agreement. Because of their high inactivating ability, β-lactamase-producing gram-negative bacteria may sometimes cause important problems in clinical practice. They prevent the antibiotic treatment directly (as being the causal bacteria) or indirectly (even when they were not the causal bacteria) in some clinical situations.[8] The "double disc method" may for this reason be acceptable as an useful tool for the detection of β-lactamase-producing bacteria in the laboratory.

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