Effect of Storage of Mueller-Hinton Agar Plates on Zone Sizes for Antimicrobial Testing

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The length of time Mueller-Hinton agar plates can be stored at 4 C without affecting the size of zones of inhibition in susceptibility testing by the Bauer-Kirby method was studied. It was found that these plates can be stored for 3 weeks at 4 C without an appreciable affect on zone sizes. Storage of plates in sealed plastic bags did not alter the results significantly. The findings indicate that commercially prepared Mueller-Hinton agar plates, which may be several days old when received at the laboratory, are suitable for use in routine susceptibility tests by the Bauer-Kirby method.

The Bauer-Kirby method is becoming increasingly acceptable as a practical method of testing antimicrobial agents in clinical laboratories. One aspect of the method which appears to have received little attention is the length of time agar plates can be stored without affecting the size of the zones of inhibition. In describing their method, Bauer and Kirby (1) recommended that Mueller-Hinton agar plates be used within 4 days of preparation. Because commercially prepared plates may be older than that when received at the laboratory, this study was undertaken to determine whether storage of Mueller-Hinton agar plates for longer than 4 days affects zone sizes and whether storage in sealed plastic bags will lengthen the shelf life of the plates. Four test organisms were used to test plates after storage for various periods of time to determine the length of time bagged and unbagged agar plates could be stored at 4 C without causing an average difference of 2 mm in zone diameters, the generally accepted normal variation in zones of inhibition. In addition, 126 clinical cultures were tested simultaneously on freshly prepared plates and on plates that had been stored for 22 days to determine whether the results were applicable to a greater variety of organisms.

MATERIALS AND METHODS

Agar. Mueller-Hinton agar (Difco no. 0252), approximately 20 ml per 100-mm plastic petri dish (Colab no. 11-205C), was used. When blood-agar was required, 5% defibrinated sheep blood was added.

Storage. Plates prepared in the laboratory as described above were stored at 4 C in a walk-in refrigerated room with circulating air (Forma Scientific Co., Marietta, Ohio). Plates were stored in the regular manner or after being placed in plastic (poly-ethylene) bags and sealed. Plates were also purchased from Hyland Labs, Los Angeles, Calif., and stored in the original Mylar bags in packages of 20 plates each, as received. All batches of plates from each source were tested at intervals, up to 24 days in some cases.

Organisms. Shigella flexneri ATCC 9216, Staphylococcus aureus ATCC 6538P, and strains of Escherichia coli and Streptococcus faecalis isolated from clinical specimens at the Hospital of the University of Pennsylvania were used.

After it was determined that 3-week storage of the plates did not affect zone sizes with stock organisms, 126 cultures were selected at random from the microbiology laboratory of the Hospital of the University of Pennsylvania and were tested simultaneously with 3-week-old and freshly prepared plates to determine whether the results were applicable to a wide variety of organisms and antimicrobial agents. The 126 cultures included E. coli (38 strains), Klebsiella spp. (20 strains), Proteus mirabilis (12 strains), Pseudomonas spp. (11 strains), Enterobacter spp. (9 strains), S. aureus (10 strains), enterococci (25 strains), and Providencia group (1 strain).

Discs. High-concentration Bacto-Sensitivity Disks were used as recommended by the Bauer-Kirby method. E. coli and S. flexneri were tested against ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), colistin sulfate (10 μg), kanamycin (30 μg), polymyxin B (300 units), and tetracycline (30 μg); S. aureus was tested against bacitracin (10 units), kanamycin, lincomycin (2 μg), nafcillin (1 μg), sodium oxacillin (1 μg), streptomycin (10 μg), and vancomycin (30 μg); and S. faecalis was tested against ampicillin, bacitracin, cephalothin, chloramphenicol, kanamycin, potassium penicillin G (10 units), and vancomycin. The above drugs were selected on the basis of their ability to produce clear-cut zones of inhibition with the appropriate organisms. The 126 clinical cultures were tested with gentamicin (10 μg), nalidixic acid (30 μg), and nitrofurantoin (300 μg), in addition to the drugs mentioned above.
Procedure. The Bauer-Kirby high-concentration disc method for antimicrobial susceptibility testing was used. Plates were inoculated in triplicate. A separate cotton swab was used for each plate, and the antimicrobial discs were applied in the usual manner. After overnight incubation, all plates were read by two persons independently, each individual making a single measurement of each zone of inhibition on the three plates. In this way, each antimicrobial agent was tested with three discs and each zone of inhibition was measured twice. The average of the six readings was recorded. This type of experiment was repeated several times; however, because the conclusions were essentially the same, the results of only one experiment are reported in detail. Zone sizes were measured by comparing the zones of inhibition with a chart of ruled circles 8 to 29 mm in diameter. When the zone exceeded 29 mm in diameter, a Vernier caliper was used.

RESULTS

In Table 1 are presented the results obtained with one large lot of Mueller-Hinton plates stored unbagged and tested over a period of 22 days with E. coli, S. flexneri, and S. aureus against seven antimicrobial drugs. S. faecalis was tested in a similar manner except that the Mueller-Hinton medium contained 5% sheep blood and the plates were tested over a period of

| Table 1. Average sizes of zones of inhibition on laboratory-prepared Mueller-Hinton agar plates after storage for periods up to 2 or 27 days |

| Organism      | Drug   | Zone size (mm) |
|---------------|--------|----------------|
|               |        | 0 days | 1 day | 4 days | 12 days | 22 days |
| Escherichia coli | Ampicillin | 17.7   | 18.3  | 16.8  | 17.8  | 18.0  |
|                | Cephalothin | 17.2   | 19.1  | 17.8  | 18.8  | 20.0  |
|                | Chloramphenicol | 22.3  | 22.8  | 22.2  | 24.1  | 23.8  |
|                | Colistin | 12.2   | 13.0  | 12.5  | 13.5  | 13.8  |
|                | Kanamycin | 21.1   | 20.0  | 19.0  | 21.8  | 20.3  |
|                | Polymyxin B | 13.8   | 14.2  | 14.3  | 14.5  | 14.1  |
|                | Tetracycline | 18.5   | 18.0  | 17.3  | 18.1  | 19.3  |
|                | Overall averages | 17.6   | 17.9  | 17.1  | 18.4  | 18.4  |
| Shigella flexneri | Ampicillin | 16.5   | 18.7  | 18.3  | 20.3  | 18.5  |
|                | Cephalothin | 20.8   | 21.1  | 20.8  | 22.3  | 22.7  |
|                | Chloramphenicol | 28.8  | 29.3  | 28.7  | 30.2  | 30.3  |
|                | Colistin | 14.8   | 15.5  | 15.1  | 15.3  | 15.2  |
|                | Kanamycin | 18.3   | 16.5  | 16.5  | 17.1  | 17.5  |
|                | Polymyxin B | 16.3   | 16.2  | 16.2  | 16.8  | 17.1  |
|                | Tetracycline | 24.7   | 23.5  | 23.7  | 23.0  | 24.0  |
|                | Overall averages | 20.4   | 20.1  | 19.9  | 20.7  | 20.8  |
| Staphylococcus aureus | Bacitracin | 23.1   | 21.7  | 21.8  | 23.7  | 21.8  |
|                | Kanamycin | 21.5   | 21.5  | 21.8  | 22.2  | 22.5  |
|                | Lincomycin | 21.5   | 20.7  | 20.7  | 20.5  | 22.2  |
|                | Nafcillin | 17.8   | 17.8  | 18.5  | 19.5  | 19.5  |
|                | Oxacillin | 19.8   | 19.5  | 20.5  | 18.7  | 20.3  |
|                | Streptomycin | 17.8   | 19.0  | 18.8  | 18.5  | 19.0  |
|                | Vancomycin | 18.7   | 19.8  | 18.3  | 19.8  | 20.0  |
|                | Overall averages | 20.1   | 20.0  | 20.0  | 20.4  | 20.8  |
| Streptococcus faecalis* | Ampicillin | 19.2   | 19.5  | 21.1  | 21.1  | 19.8  |
|                | Bacitracin | 19.1   | 16.5  | 18.1  | 18.5  | 17.7  |
|                | Cephalothin | 16.2   | 16.5  | 14.5  | 15.3  | 14.5  |
|                | Chloramphenicol | 20.0  | 20.5  | 20.5  | 18.8  | 18.8  |
|                | Kanamycin | 15.2   | 14.5  | 15.4  | 13.7  | 13.5  |
|                | Penicillin | 20.2   | 19.8  | 18.2  | 18.5  | 18.4  |
|                | Vancomycin | 16.8   | 16.8  | 17.8  | 15.8  | 17.8  |
|                | Overall averages | 18.1   | 17.6  | 17.9  | 17.4  | 17.2  |

* Tested on Mueller-Hinton agar with 5% defibrinated sheep blood.
Table 2. Average sizes of zones of inhibition on commercially prepared Mueller-Hinton agar plates after storage in sealed plastic bags for periods up to 24 days

| Organism       | Drug       | Zone size (mm) |
|----------------|------------|----------------|
|                |            | 1 day | 5 days | 10 days | 21 days | 24 days |
| **Escherichia coli** |           |       |       |         |         |         |
| Ampicillin     | 17.2       | 18.8  | 19.1  | 18.3    | 19.2    |         |
| Cephalothin    | 19.5       | 20.8  | 20.8  | 19.0    | 20.8    |         |
| Chloramphenicol | 24.3       | 25.8  | 25.3  | 25.0    | 25.3    |         |
| Colistin       | 12.8       | 12.8  | 12.7  | 12.5    | 12.0    |         |
| Kanamycin      | 21.5       | 23.2  | 22.8  | 21.3    | 22.5    |         |
| Polymyxin B    | 13.5       | 14.3  | 14.7  | 13.5    | 14.0    |         |
| Tetracycline   | 21.7       | 21.5  | 22.1  | 21.2    | 22.8    |         |
| Overall averages | 18.6    | 19.6  | 19.6  | 18.7    | 19.5    |         |
| **Staphylococcus aureus** |           |       |       |         |         |         |
| Bacitracin     | 20.5       | 16.3  | 20.5  | 20.0    | 17.5    |         |
| Kanamycin      | 22.8       | 24.5  | 24.8  | 23.5    | 23.5    |         |
| Lincomycin     | 22.3       | 21.5  | 21.8  | 23.0    | 21.3    |         |
| Nafcillin      | 19.5       | 19.1  | 19.3  | 19.8    | 20.8    |         |
| Oxacillin      | 21.5       | 20.3  | 22.3  | 20.5    | 22.0    |         |
| Streptomycin   | 20.5       | 21.1  | 21.5  | 20.2    | 20.8    |         |
| Vancomycin     | 19.5       | 21.5  | 21.3  | 19.7    | 20.3    |         |
| Overall averages | 20.9    | 20.6  | 21.6  | 21.0    | 20.9    |         |
| **Shigella flexneri** |           |       |       |         |         |         |
| Ampicillin     | 19.5       | 21.1  | 20.5  | 21.1    | 19.3    |         |
| Cephalothin    | 22.5       | 24.5  | 22.7  | 24.5    | 22.5    |         |
| Chloramphenicol | 29.8       | 31.0  | 31.0  | 29.7    | 29.2    |         |
| Colistin       | 15.5       | 16.5  | 16.8  | 15.0    | 16.1    |         |
| Kanamycin      | 17.5       | 19.0  | 17.8  | 18.8    | 17.2    |         |
| Polymyxin B    | 16.3       | 17.5  | 16.2  | 16.5    | 17.8    |         |
| Tetracycline   | 23.5       | 24.8  | 25.2  | 24.5    | 24.5    |         |
| Overall averages | 20.7    | 22.1  | 21.5  | 21.4    | 20.9    |         |

27 days. The zone sizes represent the average of six measurements for each drug. No noteworthy change was noticed when the overall average of the readings with all drugs at each test period was made.

Similar results were obtained with laboratory-prepared plates stored in sealed plastic bags and tested at intervals comparable to those used for the unbagged plates employed in the tests summarized in Table 1.

The data in Table 2 show that commercially prepared plates gave results comparable to those obtained with plates prepared in the laboratory.

There was surprisingly little difference in the sizes of the zones of inhibition on Mueller-Hinton medium in freshly prepared plates or in plates that had been stored for 21 days (Table 3). The average sizes of the zones represent two measurements on each of three discs for each drug. The average diameters of the zones of inhibition varied only 0.5 to 2.0 mm between fresh and old plates, and in no case was the variation great enough to alter the interpretation of the reactions as susceptible, intermediate, or resistant.

Although the plates used for obtaining the results listed in Tables 1 and 2 were from different sources, and although those used in connection with Table 1 were stored as unbagged plates for 24 days and those used in connection with Table 2 were stored as bagged plates for 22 days, the results were comparable for E. coli, S. flexneri, and S. aureus. Only in the cases of E. coli with kanamycin and tetracycline and S. aureus with bacitracin did the sizes of the zones of inhibition vary more than 2 mm, and these instances are discussed below.

**DISCUSSION**

Results of this study indicate that storage for at least 3 weeks at 4 C does not appreciably affect the size of the zones of inhibition on Mueller-Hinton agar plates in the Bauer-Kirby method of antimicrobial susceptibility testing. No critical cut-off time for storage of plates was observed. After 3 weeks of storage, there was some increase in variation with some of the organisms, and the results became slightly more difficult to interpret. However, in almost every case storage for 1 month or more still produced results within a safe range of variation.
| Drug            | S. aureus (10) | E. coli (35) | Klebsiella (20) | P. mirabilis (12) | Pseudomonas (11) | Entrobacter (9) | Enteroxoccus (35) | Providencia (1) |
|----------------|----------------|--------------|----------------|-------------------|------------------|----------------|------------------|-----------------|
|                | F<sup>a</sup> | O<sup>b</sup> | F  | O  | F  | O  | F  | O  | F  | O  | F  | O  | F  | O  |
| Ampicillin     | 12.9           | 12.6         | 18.0           | 17.5             | 10.0             | 9.0           | 23.8           | 24.6             | 6.0             | 6.0           | 18.6         | 18.6         | 22.6           | 22.9             | 6.0             | 6.0           |
| Bacitracin     | 18.8           | 18.8         | 21.4           | 21.4             | 18.0             | 17.5           | 22.7           | 22.7             | 6.0             | 6.0           | 20.6         | 20.3         | 16.2           | 16.7             | 6.0             | 6.0           |
| Cephalothin    | 18.0           | 18.0         | 21.4           | 21.4             | 18.0             | 17.5           | 22.7           | 22.7             | 6.0             | 6.0           | 20.6         | 20.3         | 16.2           | 16.7             | 6.0             | 6.0           |
| Chloramphenicol| —              | —            | 23.8           | 24.5             | 23.6             | 23.6           | 23.5           | 24.2             | 6.0             | 6.0           | 24.1         | 24.1         | 21.6           | 21.6             | 6.0             | 6.0           |
| Colistin       | —              | —            | 14.0           | 14.0             | 13.8             | 13.8           | 6.0            | 6.0              | 12.9            | 12.9          | 13.6         | 13.4         | 6.0            | 6.0              | 6.0             | 6.0           |
| Gentamicin     | —              | —            | 21.4           | 21.3             | 20.9             | 21.1           | 22.3           | 22.4             | 17.1            | 17.1          | 21.4         | 20.7         | 19.3           | 19.7             | 17.0            | 17.0           |
| Kanamycin      | 13.6           | 13.0         | 20.9           | 20.6             | 20.6             | 20.8           | 21.8           | 21.4             | 9.6             | 9.0           | 21.2         | 21.2         | 17.0           | 16.7             | 18.0            | 18.0           |
| Nalidixic acid | —              | —            | 23.8           | 23.8             | 19.3             | 19.4           | 21.9           | 23.0             | 11.0            | 12.0          | 24.0         | 25.3         | 6.0            | 6.0              | 23.0            | 24.0           |
| Nitrofurantoin | —              | —            | 22.2           | 22.2             | 19.0             | 19.0           | 11.9           | 13.9             | 6.0             | 6.0           | 6.0          | 6.0          | 21.4           | 21.5             | 6.0             | 6.0           |
| Oxacillin      | 20.1           | 20.3         | —              | —                | 15.5             | 15.5           | 15.0           | 15.3             | 6.0             | 6.0           | 15.7         | 15.7         | 14.9           | 21.6             | 21.6            | 6.0             |
| Penicillin     | 21.6           | 20.7         | —              | —                | 15.5             | 15.5           | 15.3           | 15.3             | 6.0             | 6.0           | 15.7         | 15.5         | 15.0           | —                | 6.0             | 6.0           |
| Polymyxin B    | —              | —            | 18.1           | 17.2             | 13.0             | 15.0           | 15.3           | 15.3             | 18.6           | 18.7          | 10.0         | 10.0         | 15.5           | 15.0             | —                | 6.0             |
| Streptomycin   | —              | —            | 18.7           | 18.7             | 19.0             | 18.6           | 6.0            | 6.0              | 6.0             | 6.0           | 17.3         | 16.8         | 15.7           | 15.4             | 6.0             | 6.0           |
| Tetracycline   | 18.8           | 18.8         | —              | —                | 19.0             | 18.6           | 6.0            | 6.0              | 6.0             | 6.0           | 17.3         | 16.8         | 15.7           | 15.4             | 6.0             | 6.0           |

* Maximal difference between fresh and O<sup>c</sup>.  
* F<sup>a</sup>: freshly prepared plates; used within 24 hr of preparation.  
* O<sup>b</sup>: old plates; unwrapped plates used after storage at 4 C for 3 weeks.  
* Tested on Mueller-Hinton agar with 5% defibrinated sheep blood.  
* No zone of inhibition; 6 mm is the diameter of the medicated discs.

The storage of plates in sealed plastic bags did not change the results to any significant extent. Apparently the loss of moisture from the medium in unbagged plates is not sufficient to produce an important change in the sizes of the zones of inhibition. One would expect that if the age of the plates was affecting the zones of inhibition the size would show a tendency to decrease or increase with age. This was not observed with the drugs tested. The stock culture of S. aureus showed a difference of 3 mm in zone sizes with bacitracin between plates stored for 1 day and plates stored for 24 days in the case of commercially prepared plates, but the difference was only 1.3 mm between laboratory plates that were freshly prepared and those stored for 22 days. One must take into consideration that the commercially prepared plates were 4 days old when received at the laboratory prior to the beginning of storage. Less variation was observed with bacitracin on fresh and stored plates in the case of the 10 recently isolated strains of S. aureus. For some unexplained reason, the results were a little more erratic at two of the five test periods with this antibiotic and the commercially prepared plates. With S. faecalis and bacitracin on laboratory-prepared plates, there was an unaccountable variation of 2.6 mm in zone sizes on plates after storage for 2 days as compared with fresh plates. A difference of this magnitude was not observed in plates of the same lot which were tested over longer periods of storage, and it did not occur when the test was repeated. In the case of E. coli and cephalothin on laboratory-prepared plates, there was a difference of 2.8 mm in zone sizes between fresh and 22-day-old laboratory-prepared plates, but the variation on commercially prepared plates was 1.3 mm.

With S. flexneri and ampicillin, the results were not quite as uniform with laboratory-prepared plates as with commercially prepared plates. On the other hand, the results with S. aureus and kanamycin were a little more uniform with laboratory-prepared plates than with commercially prepared plates.

The variations cited above were not consistent because they did not occur when the experiments were repeated. This leads to the conclusion that such variations are due to some factor other than the age of the plates.

There was an impressive lack of variation in the
results obtained when fresh and stored plates were used with recently isolated clinical cultures, and it is with cultures of this type that one is primarily concerned when using the Bauer-Kirby method. Perhaps stock cultures are less homogeneous than the recently isolated cultures, and this may account for the occasional slight variations obtained with stock cultures.

**LITERATURE CITED**

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