Rapid Method for Determining Antimicrobial Susceptibility of *Haemophilus influenzae*

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A method was developed to determine the susceptibility of *Haemophilus influenzae* to ampicillin, cefamandole, and chloramphenicol by using the MS-2 system (Abbott Laboratories) for determining minimum inhibitory concentrations (MIC). The MS-2 results for 132 strains of *H. influenzae* were compared with the results of agar disk diffusion, agar dilution, and beta-lactamase tests. Twenty-four strains (18.2%) of *H. influenzae* were resistant to ampicillin by the agar dilution method, as opposed to 25 strains by the MS-2 method. For a beta-lactamase-negative strain, the agar dilution MIC was 4 μg/ml, and the MS-2 MIC was 16 μg/ml. Twenty-one strains produced beta-lactamase; two beta-lactamase-negative strains were resistant by MS-2, agar dilution, and agar disk diffusion. In addition, one beta-lactamase-negative strain, for which the agar dilution MIC was 32 μg/ml and the MS-2 MIC was 16 μg/ml, was sensitive by agar disk diffusion. Overall, the MS-2 method compared favorably with the agar dilution method for determining the MIC of ampicillin, cefamandole, and chloramphenicol for *H. influenzae*.

**Since Haemophilus influenzae may be resistant to ampicillin, it is essential that the organism be tested in the routine diagnostic laboratories of hospitals. Chemical tests for beta-lactamase production served this purpose until it was realized that resistance was not always mediated by this enzyme (2). Thus, it became necessary to develop alternative methods for antimicrobial susceptibility testing of this organism, and both chemical and microbiological methods have been described by Thornsberry and colleagues (3, 5). A rapid method for testing the minimum inhibitory concentration (MIC) of ampicillin against *H. influenzae* by using the Autobac system was described by other workers (1). The purpose of the present study was to develop a rapid susceptibility test of *H. influenzae* to ampicillin, chloramphenicol, and cefamandole.**

**MATERIALS AND METHODS**

**Organisms.** A total of 132 isolates of *H. influenzae* were tested against ampicillin, cefamandole, and chloramphenicol by the agar dilution, agar disk diffusion, and MS-2 methods. These isolates were obtained from a variety of clinical specimens, including cerebrospinal fluid, blood, and respiratory and genital tract material. Some of the isolates were generously donated by Peter Fleming, Hospital for Sick Children, Toronto, Ontario, Canada. Isolates were maintained in 5% sheep blood containing 1% glycerol and 0.5% dimethyl sulfoxide. An ampicillin-sensitive control strain of *H. influenzae* (ATCC 9795) and a beta-lactamase-producing resistant strain were run with all sensitivity test methods.

**Agar dilution method.** Ampicillin (Bristol Laboratories), chloramphenicol (Parke, Davis & Co.), and cefamandole (Eli Lilly & Co.) were dissolved in distilled water to give stock solutions of 4.000 μg/ml. From these, doubling solutions of ampicillin, cefamandole, and chloramphenicol, ranging from 16 to 0.06 μg/ml, were incorporated into Mueller-Hinton agar supplemented with 1% hemoglobin (Difco Laboratories) and 1% IsoVitaleX (BBL Microbiology Systems), and the test was carried out as described by Thornsberry (3). Antibiotic-free plates were used as growth controls. Incubation was overnight at 35°C, and the MIC was the lowest concentration that completely inhibited growth. In the case of ampicillin, a MIC of 8 μg/ml indicated resistance; a MIC of 4 μg/ml indicated resistance in the case of both chloramphenicol and cefamandole.

**Iso-Sensitest broth (Oxoid Ltd.) was used to prepare the bacterial inoculum. A suspension that gave 0.1 absorbance at 540 nm in a spectronic 21 spectrophotometer (Bausch & Lomb, Inc.) contained 10⁶ colony-forming units (CFU) per ml. This was confirmed repeatedly by viable bacteria counts. A 10-fold dilution of this suspension was the broth used for the agar dilution test, and a Steers replicator was used to transfer 10⁴ CFU per spot inoculum.**

**Agar disk diffusion.** The agar disk diffusion test was performed by using paper disks containing 10 μg of ampicillin, 30 μg of chloramphenicol, and 30 μg of cefamandole (BBL). The inoculum, standardized to 0.5 McFarland (10⁴ CFU/ml), was streaked in three directions by using a cotton-tipped swab dipped in broth, with excess fluid expressed. The disks were applied firmly when the surface of the plate was dry. After incubation at 35°C for 24 h, the zone sizes were measured with calipers and recorded. The zone diame-
ters denoting susceptibility to ampicillin, cefamandole, and chloramphenicol were equal to or greater than 20, 18, and 18 mm, respectively; resistance was indicated by zones equal to or less than 19, 14, and 12 mm, respectively.

Beta-lactamase tests. Beta-lactamase tests were performed on all isolates by using Betatext Strips (Medical Wire & Equipment Co.) and confirmed by the Nitrocefin (Glaxo Group Research Ltd.) test. Beta-lactamase-positive and -negative strains of H. influenzae were also used.

Selection of broth for MS-2 testing. To use the MS-2 system, it was necessary to select a broth that would reliably support the growth of H. influenzae. Iso-Sensitest broth (Oxoid), Mueller-Hinton broth (BBL), Trypticase soy broth (BBL), and brain heart infusion (BBL) were supplemented with 1% and 5% Fildes extract (Difco), 1% supplement VX (Difco), 1% CVA enrichment (GIBCO), and 2% IsoVitaleX (BBL). Growth curves were plotted to determine the most suitable medium for susceptibility testing with the MS-2.

MS-2 tests for antimicrobial susceptibility. The research system of the MS-2 instrument was used to determine susceptibility by producing graphic displays of growth kinetics of bacteria in the presence of ampicillin, cefamandole, and chloramphenicol in doubling dilutions of from 16 to 0.06 μg/ml. Inoculated and un-inoculated antibiotic-free control broths were incorporated in all test runs and served as controls. Iso-Sensitest broth with 5% Fildes extract and the concentrations of antibiotics stated above was dispensed in 1.0-ml volumes in research cuvette wells. To each well was added 10 μl of bacterial inoculum to give a final inoculum of 10^5 CFU/ml. The results of tests as shown by growth kinetic curves were examined at 5 h, at which time those indicating inhibition were easily distinguished from early logarithmic curves showing bacterial growth.

MS-2 antimicrobial susceptibility tests program. All strains were tested in the antimicrobial susceptibility tests program with all possible permutations of 2.5- and 5.0-μg ampicillin disks and gram-positive, gram-negative, or alternate programs.

Reproducibility of findings. Eight strains, including ATCC 9795, were tested against all three antimicrobial agents in the three test systems daily for 5 days. Zone sizes and MIC results were recorded and compared.

RESULTS

Growth in all media containing supplement VX, CVA enrichment, or IsoVitaleX was either not detected or was unsatisfactory. The medium selected for the MS-2 studies was Iso-Sensitest broth with 5% Fildes extract added. This was based on the standardized nature of the base medium and excellent growth at 6 h, as shown in Fig. 1, with inocula of 10^1 to 10^5 CFU/ml. Typical growth curves of a strain of H. influenzae in the presence of various concentrations of ampicillin are shown in Fig. 2; they indicate a clear-cut MIC at 4 h.

Table 1 shows that the distribution of MICs in the two test procedures was similar, all strains being sensitive to chloramphenicol and cefamandole at concentrations that are readily achievable in serum. All strains were inhibited by 4 μg/ml of each antibiotic in the agar dilution tests, and growth curves were flat at this concentra-

FIG. 1. Growth curves of a strain of H. influenzae run in duplicate at concentrations of 10^1 to 10^5 CFU/ml.
tion in the MS-2. The zone sizes in the agar disk diffusion test ranged from 22.6 to 43 mm in cefamandole tests and from 26.7 to 40 mm in chloramphenicol tests.

Taking an MIC of 8 μg/ml to indicate resistance, 24 strains (18.2%) of *H. influenzae* were resistant to ampicillin by agar dilution and 25 (18.9%) by the MS-2 method (Table 1). Of these, 21 produced beta-lactamase in repeated testing. There was a near-perfect correlation between the results obtained by agar dilution and the MIC growth curves in the MS-2. The one exception was a beta-lactamase-negative strain with an agar dilution MIC of 4 μg/ml and an MS-2 MIC of 16 μg/ml.

The results of agar disk diffusion tests correlated well with the MIC methods. However, one beta-lactamase-negative strain that was repeatedly resistant by agar dilution (MIC, 32 μg/ml) was sensitive by agar disk diffusion.

Two beta-lactamase-negative strains were resistant by all three methods.

Analysis of discrepant results of MIC testing of 132 strains to the extent of two or more antibiotic dilutions is shown in Table 2. The most striking feature in this analysis was that, of all the strains that were susceptible by all three methods, the MICs generated in the MS-2 system were two to three dilutions lower than in agar in 23 of 132 tests of cefamandole, in contrast to the few discrepancies in tests of ampicillin and chloramphenicol.

Tests of *H. influenzae* strains with 2.5- and 5.0-μg ampicillin disks in all permutations of drugs and the antimicrobial susceptibility tests program (gram positive, gram negative, and alternate) proved quite unreliable, almost all of the 132 strains being reported resistant or intermediate in all methods.

In tests of reproducibility on five repeat testings, all strains gave identical results up to the limits of two antibiotic dilutions.

**DISCUSSION**

A minor disadvantage of the MS-2 system is its inability to test fastidious organisms. It was the purpose of this study to attempt to develop a test method for *H. influenzae* that would give more rapid results than the current agar disk diffusion and dilution tests. Ampicillin, cefaman-

![FIG. 2. Growth curves of a strain of *H. influenzae* in the presence of various concentrations of ampicillin. MIC = 1.0 μg/ml.](image)

**TABLE 1. Distribution of MICs for 132 strains tested against ampicillin, cefamandole, and chloramphenicol in agar dilution and MS-2**

<table>
<thead>
<tr>
<th>Antibiotic and test</th>
<th>No. of MICs of (μg/ml):</th>
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<tbody>
<tr>
<td></td>
<td>&lt;0.25</td>
</tr>
<tr>
<td><strong>Ampicillin</strong></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>0</td>
</tr>
<tr>
<td>MS-2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Chloramphenicol</strong></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>1</td>
</tr>
<tr>
<td>MS-2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Cefamandole</strong></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>0</td>
</tr>
<tr>
<td>MS-2</td>
<td>3</td>
</tr>
</tbody>
</table>

*AD, Agar dilution. The modal MICs are in bold type.*

<http://jcm.asm.org/>
dilution, and chloramphenicol were chosen as test antibiotics.

Early experiments confirmed that 5% Fildes extract in Iso-Sensitest broth gave the best growth, outperforming less optically dense media containing X and V factors. The results showed a high degree of correlation between the MS-2 broth dilution results and those derived by agar dilution, except that there were more cefamandole MIC discrepancies at the level of two antibiotic dilutions or greater. This phenomenon may be explained by the use of a higher inoculum in agar dilution tests than in the MS-2.

One beta-lactamase-negative strain was judged sensitive by the disk diffusion test and resistant by the other two techniques, suggesting that the disk diffusion method was in error. The MS-2 called a beta-lactamase-negative organism resistant (MIC, 16 µg/ml) when its MIC by agar dilution was 4 µg/ml, a "major discrepancy" by the criteria of Thornsberry and co-workers (4). However, taking an ampicillin MIC of 4 µg/ml to indicate moderate susceptibility of *H. influenzae* (6), this discrepancy would be "minor," as would be the case with the only other isolate for which the MIC was 4 µg/ml.

Despite the failure of the existing antimicrobial susceptibility tests program to provide correlating susceptibility data on our test bacteria, use of the MS-2 system gave reliable results in 5 h. It is recommended as a convenient method for the detection of that small group of beta-lactamase-negative, ampicillin-resistant, strains of *H. influenzae* and for tests of chloramphenicol and cefamandole sensitivity.

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LITERATURE CITED


