In Vitro Antibacterial Activity and Effect of Agar Medium Utilized in Its Susceptibility Testing

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The in vitro activity of minocycline against 1,028 bacterial strains was determined in parallel in Mueller Hinton Agar and Trypticase Soy Agar. The broad antibacterial effect of minocycline against gram-positive cocci and gram-negative bacilli is confirmed. Minimal inhibitory concentrations for gram-positive bacteria in Mueller Hinton Agar were at least twofold less than in Trypticase Soy Agar. Minimal inhibitory concentrations for gram-negative bacilli in Mueller Hinton Agar were usually fourfold less than in Trypticase Soy Agar.

Minocycline is the semisynthetic tetracycline derivative 2-dimethylamino-6-deoxy-6-demethyl-tetracycline. This report is designed to serve two purposes: first, to amplify on the available information on susceptibility of potentially pathogenic bacteria, especially gram-negative bacilli, to this agent, and, second, to demonstrate the difference in minimal inhibitory concentrations (MIC) obtainable in two different agar media.

MATERIALS AND METHODS

Minocycline powder in 100-mg amounts in sterile vials was used.

With the exception of a few stock laboratory strains of uncommonly isolated bacteria, the bacteria studied represented fresh clinical isolates identified in the General Bacteriology Laboratory of the Mayo Clinic. Members of the family Enterobacteriaceae were identified with methods described by Edwards and Ewing (1) and classified according to Ewing (2). Nonfermenting gram-negative bacilli were identified and classified by the method of King (6). Staphylococci were specified according to tube coagulase activity by using Difco Coagulase Plasma. Streptococcus pyogenes was presumptively identified by susceptibility to bacitracin (Taxo disc, BBL). Group D streptococci were identified with bile-esculin agar (9; R. R. Facklam and M. D. Moody, Bacteriol. Proc., p. 72, 1969). Strains of Haemophilus were specified according to X and V factor (BBL) requirements and hemolysis on Horse Blood Agar (BBL).

Susceptibility testing was carried out by the agar-dilution technique by using the replicator apparatus of Steers et al. (4, 7). Antibiotic was added to agar at 50 to 55 C to give the appropriate concentrations in a twofold dilution series. The agar was poured into 100- by 15-mm square, disposable plastic dishes to a depth of 2.5 to 3 mm, and allowed to harden on a flat surface before storage at 4 C. Inoculum size was adjusted by visual comparison with a McFarland standard so as to contain a final concentration of approximately 10^6 viable units on the surface of the agar. All determinations were carried out in parallel in both Trypticase Soy Agar (TSA,BBL) and Mueller Hinton Agar (MHA, BBL). Sheep blood in a final concentration of 4% was added to both of the agar media for testing of the Viridans group of streptococci, S. pyogenes, Diplococcus pneumoniae, Brucella sp., Pasteurella sp., and Listeria monocytogenes. Haemophilus sp. were tested on Chocolate Blood Agar with Yeast Extract (Isovitalex, BBL) and incubated in an environment of 10% carbon dioxide. Incubation time was 16 to 18 hr at 37 C. MIC was read at the concentration at which there was no visible growth, a very fine barely visible growth, or a single colony.

Each set of plates included Staphylococcus aureus (ATCC 14777) with a stable MIC to minocycline as control. One plate of each type of agar without antibiotic was inoculated as a control.

RESULTS

A total of 1,028 bacterial strains was tested. With the exception of Haemophilus sp., all strains were tested in parallel on MHA and TSA. Cumulative susceptibility curves were drawn for groups of organisms with greater than 50 test strains. Results of testing of groups with 15 to 50 test strains were plotted in a stepwise cumulative fashion, and results of testing of groups with less than 15 test strains were tabulated.

All of the 108 strains of S. aureus were inhibited by 3.12 and 6.25 µg of minocycline per ml in MHA and TSA, respectively (Fig. 1). Although
TABLE 1. Relationship of minimal inhibitory concentrations (MIC) of minocycline to gram-positive bacteria in Mueller Hinton Agar (MHA) and in Trypticase Soy Agar (TSA).

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC range in MHA (µg/ml)</th>
<th>MIC range in TSA (µg/ml)</th>
<th>Fold relationship of MIC in TSA&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pyogenes</td>
<td>0.1-0.39</td>
<td></td>
<td>-2  0  +2  or &gt;</td>
</tr>
<tr>
<td>Viridans group of streptococcus</td>
<td>0.2-0.78</td>
<td></td>
<td>1  4  4</td>
</tr>
<tr>
<td>Diplococcus pneumoniae</td>
<td>0.2-0.78</td>
<td></td>
<td>1  4  4</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>0.1-0.2</td>
<td></td>
<td>4  11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values indicate number of strains; at minus fourfold, no strains were present.

the differences in MIC between the two media were not great at a concentration of 0.39 µg/ml or more, only 9% of the strains were inhibited by 0.2 µg/ml in TSA, whereas 85% were inhibited by the same concentration in MHA. Seventy-six per cent of the strains of Staphylococcus epidermidis were inhibited by 0.78 µg of the antibiotic per ml in TSA and 98% by 0.78 µg/ml in MHA (Fig. 1).

The group D streptococci (Fig. 1) were less sensitive than the staphylococci, with only 33% inhibited at a concentration of 3.12 µg of minocycline per ml in TSA and 39% inhibited at the same concentration in MHA. At concentrations greater than 3.12 µg/ml, the differences in MIC between the two agar media became larger.

The same pattern of apparent greater susceptibility of gram-positive organisms to minocycline in MHA has persisted, with most MIC in TSA being twofold greater (Table 1).

Seventy per cent of Escherichia coli strains were inhibited by 3.12 µg/ml in TSA and 81% by the same concentration in MHA (Fig. 2). With 100 strains of Klebsiella sp., 3.12 µg of minocycline per ml was inhibitory to 29% of the strains in TSA and to 81% in MHA (Fig. 2). Similar dif-
MINOCYCLINE SUSCEPTIBILITY TESTING

Fig. 3. Cumulative percentage of susceptibility data of Citrobacter, P. mirabilis, and other Proteus species (including P. vulgaris, P. rettgeri, and P. morganii) to minocycline in Mueller Hinton Agar (MHA) and Trypticase Soy Agar (TSA).

Fig. 4. Cumulative percentage of susceptibility data of Pseudomonas aeruginosa and Herellea vaginicola to minocycline in Mueller Hinton Agar (MHA) and Trypticase Soy Agar (TSA).

Fig. 5. Cumulative percentage of susceptibility data of Haemophilus species to minocycline in chocolate blood agar (CBA) with yeast extract.

Differences in MIC between TSA and MHA were noted with Enterobacter sp. (Fig. 2), with 88% inhibition at 3.12 μg/ml in MHA and 15% inhibition at the same level in TSA. Ninety-five per cent of 45 strains of Serratia marcescens were inhibited by 3.12 μg of the antibiotic per ml in MHA and by 12.5 μg/ml in TSA (Fig. 2). Similar differences were noted with Citrobacter (Fig. 3); 96% of the 23 strains tested were inhibited by 3.12 μg/ml in MHA and only 9% were inhibited by the same concentration in TSA.

Differences in susceptibility patterns between the two media were rather striking with Proteus mirabilis (Fig. 3), and were present but not nearly so striking with other species of Proteus (Fig. 3). In addition, P. mirabilis was less susceptible to minocycline than were the other species of Proteus.

Although Pseudomonas aeruginosa was not especially sensitive to minocycline in concentrations of 3.12 μg/ml or less, substantial differences in susceptibility occurred in the two media at concentrations of 6.25 μg/ml and more (Fig. 4). Herellea vaginicola was very susceptible to minocycline in both media, but also demonstrated far greater susceptibility in MHA than in TSA (Fig. 4).

All isolates of Haemophilus sp. were inhibited at a concentration of 3.12 μg/ml when tested on fortified Chocolate Agar only (Fig. 5). Included in these 31 strains were 9 H. influenzae, 12 H. parainfluenzae, and 10 H. parahaemolyticus. There was no species difference in susceptibility within
Table 2. Relationship of minimal inhibitory concentrations (MIC) of minocycline to gram-negative bacteria in Mueller Hinton Agar (MHA) and in Trypticase Soy Agar (TSA)

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC range in MHA (µg/ml)</th>
<th>Fold relationship of MIC in TSA(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 +2 +4 or &gt;</td>
</tr>
<tr>
<td>Shigella</td>
<td>1.5</td>
<td>2 3</td>
</tr>
<tr>
<td>Salmonella</td>
<td>0.78–3.1</td>
<td>4 4</td>
</tr>
<tr>
<td>Edwardsiella</td>
<td>0.39</td>
<td>1 1</td>
</tr>
<tr>
<td>Arizona</td>
<td>1.5</td>
<td>1 1</td>
</tr>
<tr>
<td>Pasteurella(b)</td>
<td>0.2–0.39</td>
<td>3 1 4</td>
</tr>
<tr>
<td>Brucella sp.(c)</td>
<td>0.01–0.05</td>
<td>5 4 3</td>
</tr>
<tr>
<td>Mima polymorpha</td>
<td>0.1–0.2</td>
<td>1 3</td>
</tr>
</tbody>
</table>

* Values indicate number of strains; at minus fourfold and minus twofold, no strains were present.
\(a\) Includes six strains of P. multocida, one strain each of P. gallinarum and P. pseudotuberculosis.
\(c\) Includes two strains of B. abortus, four strains of B. melitensis, and six strains of B. suis.

the genus Haemophilus. Two strains each of Neisseria gonorrhoeae and N. meningitidis were inhibited by concentrations of minocycline that ranged from 0.1 to 0.78 µg/ml.

In Table 2 are listed the comparative relationships of MIC values of various gram-negative bacilli tested in small numbers. Eight strains of Salmonella were inhibited by minocycline in concentrations of 3.1 µg/ml or less in MHA and in concentrations of 6.2 µg/ml or less in TSA. Five strains of Shigella were inhibited by concentrations of 1.5 µg/ml or less in MHA and by 6.2 µg/ml or less in TSA. The eight strains of Pasteurella were inhibited by 0.39 µg/ml or less in MHA and 1.5 µg/ml or less in TSA. The 12 strains of Brucella were inhibited by 0.05 µg/ml or less in MHA and 0.39 µg/ml or less in TSA.

**DISCUSSION**

As demonstrated in this study, the antibacterial activity of minocycline is very broad. Similar findings have been published by Steigbigel et al. (8), and by Fedorko et al. (3), although cumulative percentages of strains inhibited at any given concentration of antibiotic reported by them are lower than those percentages obtained in our study. Steigbigel et al. used an inoculum of 10⁶ organisms per ml and the agar-dilution method with Brain Heart Infusion Agar (Difco). Fedorko et al. used a similar inoculum size and the broth-dilution method with Trypticase Soy Broth. Of the two studies, the data of Fedorko et al. approximate our own data with TSA more closely than those of Steigbigel et al. Differences in results probably reflect differences in inoculum size and in test media. Our inoculum size was a full log lower than that of either of the other two studies, both of which present data on the effect of inoculum size. This factor probably accounts for most of the differences between our results in TSA and those of Fedorko et al. (3) in Trypticase Soy Broth, because in our own experience no appreciable systematic difference was noted between MIC values obtained in TSA and Trypticase Soy Broth or between MIC values obtained in MHA and Mueller Hinton Broth. The absence of a significant difference between the agar-dilution and the broth-dilution MIC values undoubtedly reflects the clear-cut end points obtained with the agar-dilution method.

The variations between our data in TSA and those of Steigbigel et al. (8) probably reflect differences in inoculum size and media. Brain Heart Infusion Agar is a rich medium that is especially recommended for the cultivation of fastidious bacteria and fungi, a characteristic not equally shared by TSA. Whether, in addition, there may be substances inhibitory to minocycline in Brain Heart Infusion Agar can only be speculated.

In nearly every instance in this study, MIC values obtained in MHA were at least one twofold dilution lower than those obtained in TSA. With many gram-negative bacilli and especially with P. aeruginosa, MIC values in MHA were frequently two dilutions or fourfold lower than those in TSA. Growth on TSA has been noted to be more luxuriant than on MHA, on which, in fact, one may not get growth of some streptococci, occasional staphylococci, and other fastidious organisms without supplementing the agar with blood. Again, one may also invoke the possibility of inhibitory factors to minocycline in TSA.

That agar may be inhibitory to activity of some antibiotics has been discussed by Hanus et al. (5). Depending therefore on the medium utilized, not counting the inoculum size, a spectrum of activity of minocycline can be obtained against various bacteria. It may indeed be invalid to compare MIC values obtained in media of differing characteristics without some alteration of inoculum size. This variability of results assumes considerable importance, since the assignment of the descriptions "sensitive" or "resistant" usually reflects whether the organism is inhibited by concentrations of antibiotic readily attainable in serum with usually recommended doses.
ACKNOWLEDGMENT
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LITERATURE CITED