Effect of NaCl Supplementation of Mueller-Hinton Broth on Susceptibility of Staphylococci to Aminoglycosides

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The addition of NaCl to cation-supplemented Mueller-Hinton broth increased the MICs of gentamicin, amikacin, and netilmicin for coagulase-positive and -negative staphylococci in a concentration-dependent manner. At 2% NaCl, geometric mean MICs were elevated 14.1- to 25.6-fold. Mueller-Hinton broth containing added NaCl should not be used for testing the susceptibility of staphylococci to aminoglycosides.

The use of cation-supplemented Mueller-Hinton broth containing 2% NaCl has recently been recommended for broth microdilution tests of staphylococcus susceptibility to semisynthetic penicillinase-resistant penicillins and cephalosporins (10). The purpose of the recommendation was to facilitate the detection of heteroresistant strains of staphylococci which exhibit a faster rate of growth in the presence of high NaCl concentrations (1, 5). Because of the growth inhibitory effect of NaCl upon bacteria other than staphylococci, the routine addition of 2% NaCl to Mueller-Hinton broth is contraindicated. However, laboratories and commercial manufacturers which prepare broth microdilution trays for the susceptibility testing of staphylococci may be considering such a practice. In the present study, we report the effect of increased concentrations of NaCl upon the susceptibility of coagulase-positive and -negative staphylococci to aminoglycoside antibiotics.

Gentamicin and amikacin susceptibility test powders were purchased from Sigma Chemical Co., St. Louis, Mo. Netilmicin was obtained from Schering Corp., Kenilworth, N.J.

The study included 104 strains of coagulase-negative and 24 strains of coagulase-positive staphylococci. Forty-eight of the coagulase-negative and all of the coagulase-positive staphylococcal isolates were resistant to gentamicin (MIC, ≥6.3 μg/ml). All strains were recently isolated from pediatric patients.

Broth dilution susceptibility studies were performed in microtiter trays with the MIC-2000 apparatus (Dynatech Laboratories, Inc., Alexandria, Va.) by the method described in the National Committee for Clinical Laboratory Standards standard M7-T (8) with modifications suggested by Thornsberry and McDougal (10). Solutions of gentamicin, amikacin, and netilmicin ranging in concentration from 0.006 to 1.600 μg/ml were prepared in Mueller-Hinton broth (lot no. 688589; Difco Laboratories, Detroit, Mich.) supplemented with 50 mg of calcium and 25 mg of magnesium per liter (9) and 0, 0.25, 0.5, 1, 2, 3, or 4% NaCl. Each set of antibiotic solutions was dispensed in 0.1-ml amounts into the wells of microtiter trays. Trays were frozen immediately and stored at -70°C until used. Thawed trays were inoculated with 0.0015 ml of suspensions of the test organisms in saline. Suspensions were prepared from overnight Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy broth cultures so that the numbers of bacteria in the wells after inoculation approximated 109 CFU/ml. Trays were incubated at 35°C for a full 24 h and examined to determine the

<table>
<thead>
<tr>
<th>% NaCl added</th>
<th>Geometric mean MIC of drugs for isolates that are:</th>
<th>Gentamicin sensitive, coagulase negative (56)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coagulase positive (24)</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>0</td>
<td>25.0</td>
<td>3.2</td>
</tr>
<tr>
<td>0.25</td>
<td>36.4</td>
<td>5.1</td>
</tr>
<tr>
<td>0.5</td>
<td>64.8</td>
<td>8.4</td>
</tr>
<tr>
<td>1</td>
<td>179.0</td>
<td>14.8</td>
</tr>
<tr>
<td>2</td>
<td>434.7</td>
<td>46.0</td>
</tr>
<tr>
<td>3</td>
<td>919.0</td>
<td>82.4</td>
</tr>
<tr>
<td>4</td>
<td>≥2,294.3</td>
<td>184.0</td>
</tr>
</tbody>
</table>

a Numbers in parentheses are numbers of strains tested.

NT, Not tested.

The study included 104 strains of coagulase-negative and
MIC of each antibiotic. The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth.

Geometric mean MICs of gentamicin, amikacin, and netilmicin for gentamicin-resistant strains of both coagulase-positive and -negative staphylococci increased in an exponential fashion with linear increases of NaCl concentration (Table 1). Regression analysis revealed very high correlations between the amount of NaCl added to Mueller-Hinton broth and the logarithms of the geometric mean MICs. The respective calculated correlation coefficients in tests of coagulase-positive and -negative staphylococci were 0.982 and 0.986 for gentamicin, 0.986 and 0.992 for amikacin, and 0.983 and 0.980 for netilmicin. At NaCl concentrations greater than 4%, growth inhibition became an increasing problem, particularly among the coagulase-negative isolates. For gentamicin-resistant staphylococci, the ratios between the geometric mean MICs in the presence and absence of 2% NaCl were 17.4 for gentamicin, 14.4 for amikacin, and 23.7 for netilmicin with coagulase-positive strains and 14.1 for gentamicin, 15.4 for amikacin, and 25.6 for netilmicin with coagulase-negative strains.

Studies on 56 isolates of gentamicin-sensitive coagulase-negative staphylococci showed that the effect of NaCl upon aminoglycoside susceptibility was not limited to gentamicin-resistant strains (Table 1). The ratios between the geometric mean MICs in NaCl-supplemented media and those containing 2% NaCl were 18.6 for gentamicin and 18.0 for netilmicin.

Tests on a different collection of aminoglycoside-sensitive and -resistant coagulase-positive and -negative staphylococci showed that the addition of 2% NaCl effected less than a twofold change in geometric mean MICs of vancomycin for all categories of isolates (data not shown). The resistant strains employed in this experiment had known aminoglycoside-resistance mechanisms. The effect of 2% NaCl on the aminoglycoside susceptibility of strains containing 4'-aminoglycoside nucleotidyltransferase or 2'-aminoglycoside phosphotransferase plus 6'-aminoglycoside acetyltransferase inactivating enzymes was the same as that seen in the sensitive strains and the resistant clinical isolates (Table 2).

Three different mechanisms may be involved in the development of resistance to aminoglycosides (3). The 3OS ribosomal subunit may be structurally altered by genetic mutation so that the affinity for antibiotic is sharply reduced, the synthesis of aminoglycoside-modifying enzymes may be directed by plasmid-borne genes, or the transport of antibiotic into the cell may be impaired by a deficiency in energy-dependent uptake. The explanation for the observed salt effect upon the susceptibility of staphylococci to aminoglycoside antibiotics is not readily obvious. Aminoglycoside resistance caused by ribosomal alteration is extremely rare among isolates from patients and is considered clinically unimportant (3). Our finding that the activities of three structurally different aminoglycosides were affected to approximately the same degree by increased salt concentration suggests that NaCl-stimulated enzymatic inactivation is unlikely, since the aminoglycoside-modifying enzymes have some measure of target specificity (2). The fact that both gentamicin-sensitive and -resistant strains were subject to the salt effect suggests that decreased transport of these antibiotics into the cells is a possible consequence of a high-ionic-strength environment.

Mates and colleagues developed a model for gentamicin uptake by *Staphylococcus aureus* in which the existence of a sufficiently large electrical potential (Δφ) across the bacterial cell membrane was necessary before antibiotic transport could occur (6, 7). They found that circumstances which decreased the magnitude of Δφ, such as anaerobiosis, presence of ionophores, or a lowering of the external pH, simultaneously decreased gentamicin uptake by cells and ultimately decreased quantitative cell death. It remains to be seen whether an elevated external salt concentration directly or indirectly results in a reduction in Δφ and a corresponding reduction in aminoglycoside uptake.

The salt effect may have a practical application in the laboratory, in that microbiological assays for antibiotic levels in which staphylococcal strains are used as indicator organisms could be performed on media containing high concentrations of NaCl. In this way patient body fluids containing an aminoglycoside as well as the antibiotic of interest could be tested without interference from the aminoglycoside. This approach toward neutralizing aminoglycoside activity would be much less expensive than incorporation of sodium polyanetholesulfonate into the assay media (4) or pretreatment of patient specimens with commercially obtained aminoglycoside-modifying enzymes.

In conclusion, the addition of NaCl to cation-supplemented Mueller-Hinton broth substantially increased the MICs of aminoglycosides for staphylococci in a concentration-dependent manner. While this practice enhances the detection of strains resistant to the semisynthetic penicillins-resistant penicillins and cephalosporins, it is not advisable for testing the aminoglycoside susceptibility of staphylococci.

(The results of this study were presented in part at the Annual Meeting of the American Society for Microbiology, Las Vegas, Nev., 3 to 7 March, 1985.)

**LITERATURE CITED**


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**TABLE 2. Effect of 2% NaCl on the aminoglycoside susceptibility of staphylococci with known aminoglycoside resistance mechanisms**

<table>
<thead>
<tr>
<th>Resistance mechanism</th>
<th>No. of strains</th>
<th>NaCl concn (%</th>
<th>Geometric mean MICs (μg/ml) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gentamicin</td>
</tr>
<tr>
<td>ANT (4')</td>
<td>6</td>
<td>0</td>
<td>2.8</td>
</tr>
<tr>
<td>PH (2') plus AAC (6')</td>
<td>10</td>
<td>2</td>
<td>32</td>
</tr>
</tbody>
</table>

⁶ The levels of resistance to netilmicin and amikacin vary with the level of 6'-acetylating activity expressed.

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**NOTES**

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