Relationship between In VitroSusceptibility Test Results for Chloramphenicol and Production of Chloramphenicol Acetyltransferase by Haemophilus influenzae, Streptococcus pneumoniae, and Aerococcus Species

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Haemophilus influenzae, Streptococcus pneumoniae, and Aerococcus species were tested for susceptibility to chloramphenicol by standard broth microdilution and disk-diffusion methods. MICs and zone diameter breakpoints were correlated with production of chloramphenicol acetyltransferase (CAT). A comparison of MICs and zone diameters indicated that the interpretative criteria for H. influenzae and S. pneumoniae should be an MIC of ≤4 μg/ml or a zone diameter ≥25 mm for susceptible strains and an MIC of ≥8 μg/ml or a zone diameter of ≥20 mm for resistant strains; for Aerococcus species, interpretative criteria should be an MIC of ≥8 μg/ml or a zone diameter of ≥20 mm for susceptible strains and an MIC of ≥32 μg/ml or a zone diameter of ≥12 mm for resistant strains. All but four strains of H. influenzae and one strain of S. pneumoniae that were resistant to chloramphenicol by these criteria produced CAT. For Aerococcus species, however, chloramphenicol-resistant strains were negative for CAT as determined by a commercially available disk test. When comparing susceptibility results with CAT production, thiampenicol was a better indicator of the presence of the enzyme than chloramphenicol and may be useful in assaying resistance to chloramphenicol.

Detecting chloramphenicol resistance in Haemophilus influenzae and Streptococcus pneumoniae when trying to relate results of in vitro susceptibility tests to chloramphenicol acetyltransferase (CAT) production has been a problem which has been of increasing relevance since chloramphenicol resistance has occurred in these species (2–5, 7). Recently, the National Committee for Clinical Laboratory Standards (NCCLS) revised the breakpoints for H. influenzae to better correlate CAT production with chloramphenicol-resistant strains of H. influenzae (12). The NCCLS did not, however, change the breakpoints for S. pneumoniae, an organism also known to produce CAT (2, 6, 7).

Even though chloramphenicol is not a drug of first choice for infections due to H. influenzae or S. pneumoniae, it is usually recommended as an alternative agent. Furthermore, susceptibility tests with chloramphenicol are almost always performed with clinical isolates of these species, and results are reported to the clinician. Since chloramphenicol could be used for therapy of infections due to these bacteria, it was important to determine whether the presently described methods were adequate to detect chloramphenicol resistance. Furthermore, it was important to determine whether breakpoints recommended for H. influenzae could be used for S. pneumoniae. Even though little information is available on therapeutic choices for infections due to Aerococcus species, we included them because studies in our laboratory had indicated that some strains were resistant to chloramphenicol, and, as with the other two species, susceptibility tests with chloramphenicol are likely to be performed and reported. Furthermore, preliminary studies had indicated that the breakpoints for Aerococcus species would have to be different from those for H. influenzae and S. pneumoniae.

Therefore, we performed studies to define interpretative criteria and to correlate the production of CAT with resistance as judged by MICs and zones of inhibition for these organisms.

MATERIALS AND METHODS

Antimicrobial agents. Chloramphenicol and thiampenicol standard powders were provided by Parke, Davis & Co., Morris Plains, N.J., and USV Pharmaceuticals Corp., Tuckahoe, N.J. Stock solutions (1.280 μg/ml) of each powder were prepared by dissolving chloramphenicol in 1 to 2 ml of ethanol and dissolving thiampenicol in 1 to 2 ml of dimethyl sulfoxide. These solutions were diluted to a concentration of 1.280 μg/ml in distilled water and filter sterilized, and 3-ml portions were stored at −70°C (11). Chloramphenicol disks (30 μg) were purchased from BBL Microbiology Systems, Cockeysville, Md.

Bacterial isolates. Clinical isolates were from several locations in the United States, Czechoslovakia, and South Africa and were stored at −70°C in defibrinated blood. A total of 234 bacterial isolates with various susceptibility patterns were tested: 113 S. pneumoniae isolates, 91 H. influenzae isolates, and 30 isolates of Aerococcus species. Staphylococcus aureus ATCC 29213 and Escherichia coli ATCC 25922 were tested as standard control strains.

Antimicrobial susceptibility testing methods. MICs were determined by broth microdilution tests according to the procedures outlined by the NCCLS (11). In brief, twofold dilutions of the study drugs in cation-supplemented Mueller-Hinton broth were tested with concentrations ranging from 0.25 to 32 μg/ml. Dilutions of 6, 10, 12, 14, 20, and 24 μg/ml were also prepared and then dispensed into microdilution panels. The test panels were stored at −70°C. When needed, the test panels were allowed to thaw at room temperature.
and were inoculated by using an inoculator (MIC 2000; Dynatech Laboratories, Inc., Alexandria, Va.). The final inoculum in each well was approximately $2 \times 10^8$ CFU per ml. The size of the inoculum was confirmed periodically throughout the study by colony counts taken directly from the growth control well of the test panel (the counts ranged from $1.9 \times 10^5$ to $3.9 \times 10^5$ CFU/ml, except for one count of $4.0 \times 10^5$ CFU/ml with a pneumococcus). The panel trays were incubated at $35^\circ C$ in 5% $CO_2$ for 16 to 18 h for S. pneumoniae and Aerococcus species and for 24 h for H. influenzae without increased $CO_2$. The MIC was recorded as the lowest concentration that inhibited visible growth of the test strain. The broth media were supplemented with 5% lysed horse blood and NAD (5 μg/ml) for testing H. influenzae or with 5% lysed horse blood for testing S. pneumoniae and Aerococcus species.

A standardized disk-diffusion susceptibility test was performed according to the method outlined by the NCCLS (10). Mueller-Hinton agar (BBL) with 5% sheep blood was used to test S. pneumoniae and Aerococcus species. Enriched Mueller-Hinton agar plates supplemented with 1% bovine hemoglobin and 1% IsoVitaleX (BBL) were used to test H. influenzae. The plates were inoculated with a swab from a suspension containing approximately 1 $\times 10^8$ CFU/ml and were incubated aerobically overnight at 35°C. S. pneumoniae and Aerococcus species were incubated for 16 to 18 h in 5% $CO_2$, and H. influenzae was incubated for 24 h without increased $CO_2$. Zone diameters were measured to the nearest millimeter with a caliper.

**Linear regression analysis.** Interpretive MICs and zone standards were obtained by comparing zones of inhibition with MICs by using both regression analysis (method of least squares) and the error-rate bounding method of Metzler and DeHaan (8).

CAT assay. A rapid tube test for CAT activity was performed according to the procedures outlined by Azemun et al. (1). A second rapid test for CAT activity was performed by using a commercially available reagent-impregnated paper disk (Remel, Lenexa, Kans.) with minor modifications to the method of the manufacturer. In brief, the strains were grown overnight on solid media impregnated with 30-μg chloramphenicol disks (for induction of the enzyme). Cells were taken from around a disk with a cotton swab to make a 1.0 McFarland standard cell suspension in 5 ml of sterile physiologic saline. Samples (0.2 ml) of the suspension were added to glass tubes (10 by 75 mm) containing Remel test and control disks. The tubes were incubated at 35°C for 30 min, and each reaction was evaluated by comparing the color in the control tube with that in the experimental tube. A color range from pale yellow to deep yellow indicated CAT activity.

**RESULTS**

The relationships between MICs and zones of inhibition for chloramphenicol obtained with isolates of Aerococcus species, H. influenzae, and S. pneumoniae were compared by regression analysis and error-rate bounding. Scattergrams for the strains tested are shown in Fig. 1.

For Aerococcus species (Fig. 1A), we suggest MIC and zone diameter breakpoints of $\geq 32$ μg/ml and $\geq 12$ mm, respectively, for resistant strains and $\leq 8$ μg/ml and $\geq 20$ mm, respectively, for susceptible strains. With these breakpoints the overall agreement was 100%, with no interpretative errors. For H. influenzae and S. pneumoniae (Fig. 1B and C), we suggest MIC and zone diameter breakpoints of $\geq 8$ μg/ml and $\geq 20$ mm, respectively, for resistant strains and $\leq 4$ μg/ml and $\leq 25$ mm, respectively, for susceptible strains. For H. influenzae, there was one (1.1%) minor error (false-intermediate) and one (1.1%) major (false-resistant) error. The results for S. pneumoniae showed two (1.8%) minor errors and one (0.8%) major error.

The susceptibility levels of Aerococcus species, H. influenzae, and S. pneumoniae to chloramphenicol are shown in Table 1. On the basis of zone size, none of the chloramphenicol-susceptible strains of H. influenzae were producers of CAT; however, one strain that was chloramphenicol-susceptible by MIC was CAT positive (CAT+). Four CAT-negative (CAT−) strains of H. influenzae were resistant by both zone size and MIC, with modes of 13 mm and 20 μg/ml, respectively. We also observed 39 CAT+ strains that were resistant by zone size, with a mode of 16 mm, and 37 CAT+ strains that were resistant by MIC, with a mode of 8 μg/ml.

For S. pneumoniae, 46 strains were both CAT− and susceptible to chloramphenicol. None of the S. pneumoniae strains with susceptible zone sizes were CAT+, but one strain with a susceptible MIC was CAT+. Of the 67 S. pneumoniae strains with resistant zone sizes (range, 9 to 18 mm; mode, 16 mm), 66 were CAT+. Of the 64 S. pneumo-
niae strains with resistant MICs (range, 8 to 14 µg/ml; mode, 10 µg/ml), 63 were CAT+.

Of 30 Aerococcus strains tested for susceptibility to chloramphenicol, 28 were susceptible by zone size and MIC, with modes of 24 mm and 6 µg/ml, respectively. None of the strains were CAT−, although two were resistant to chloramphenicol by both zone size and MIC determination.

Figure 2 shows a comparison of the MICs of thiamphenicol and thiamphenicol for CAT+ strains of S. pneumoniae and H. influenae. Of the 106 CAT+ strains tested, 66 were S. pneumoniae and 40 were H. influenae. The MICs of thiamphenicol were >32 µg/ml for the CAT+ strains of S. pneumoniae, whereas the MICs of chloramphenicol ranged from 4 to 14 µg/ml, with 95% occurring between 8 and 14 µg/ml. The MICs of thiamphenicol for all strains of H. influenae were >32 µg/ml, but only one strain had an MIC of >32 µg/ml for chloramphenicol. MICs of chloramphenicol for H. influenae strains ranged from 2 to >32 µg/ml, with 53% occurring between 8 and 16 µg/ml, and three strains (4.5%) had an MIC of ≤6 µg/ml.

The thiamphenicol MICs for all CAT− strains were ≤14.0 µg/ml, except for one strain of S. pneumoniae and two strains of Aerococcus species for which MICs were >32.0 µg/ml. The thiamphenicol modal MICs were 0.5, 2.0, and 4.0 µg/ml for H. influenae, S. pneumoniae, and Aerococcus species, respectively. Three strains of H. influenae with thiamphenicol MICs of 14 µg/ml did not produce CAT.

DISCUSSION

In 1975, the NCCLS established MIC and zone diameter breakpoints for chloramphenicol: ≥25 µg/ml and ≤12 mm, respectively, for resistant and ≤12.5 µg/ml and ≥18 mm, respectively, for susceptible (9). These breakpoints proved to be problematic when H. influenae and S. pneumoniae strains were tested for chloramphenicol resistance by relating in vitro susceptibility test results to CAT production. Recently, the NCCLS developed new MIC and zone diameter breakpoints for chloramphenicol susceptibility in H. influenae (12); ≥8 µg/ml and ≥26 mm, respectively, for resistant and ≤4 µg/ml and ≥27 mm, respectively, for susceptible. The NCCLS did not, however, establish specific breakpoints for chloramphenicol susceptibility in S. pneumoniae, even though these bacteria are also known to produce CAT (2, 12). Therefore, for organisms other than H. influenae, the breakpoints for chloramphenicol are the same as those recommended in 1975.

We studied the susceptibility of H. influenae, S. pneumoniae, and Aerococcus species to chloramphenicol and recommend new breakpoints that might better assess the relationship between CAT production and chloramphenicol resistance in these strains. We suggest that the MIC and zone diameter breakpoints for H. influenae and S. pneumoniae can be the same (≥8 µg/ml and ≥20 mm, respectively, for resistant and ≤4 µg/ml and ≥25 mm, respectively, for susceptible). However, the breakpoints for Aerococcus species must be different (≥32 µg/ml and ≤12 mm for resistant and ≤8 µg/ml and ≥20 mm for susceptible). Using these breakpoints, we had no very major errors, fewer than 1%
major errors, and fewer than 2% minor errors for *H. influenzae* and *S. pneumoniae* and no interpretative errors with *Aerococcus* species. These values are well below the 1% very major, 4% major, and 5% minor errors, a combined total of 10% errors, recommended by Thornsberry and Gavan (13).

We used two rapid methods to assay CAT production by *H. influenzae*, *S. pneumoniae*, and *Aerococcus* species: a tube assay and a disk assay using a commercially available kit. Assays of *S. pneumoniae* and *Aerococcus* species for CAT production yielded conflicting results with these two tests, but assays of CAT production in 91 *H. influenzae* isolates yielded 100% correlation. This is in contrast to the results of Doern et al. (3), who found that the tube test was significantly more accurate than the disk test in demonstrating CAT production in *H. influenzae*. The reason(s) for the differences between our results and those of Doern et al. (3) is not clear, since the same methods were used in both studies. Further studies will be necessary to clarify this problem.

Two *Aerococcus* strains were clearly resistant to chloramphenicol by zone size (9 mm and 10 mm) and MIC (32 µg/ml) but were CAT− by both methods. These strains may be resistant to chloramphenicol by a mechanism other than CAT production, or a more sensitive method for detecting CAT production may be required for this species.

When the susceptibility results obtained for chloramphenicol and thiamphenicol are compared, the thiamphenicol results are bimodal but the chloramphenicol results tend to be spread over the entire range of values. When these data are further compared with the CAT data, it becomes apparent that the bimodality of the thiamphenicol data reflects the production of CAT. Therefore, thiamphenicol susceptibility results indicate CAT production better than chloramphenicol susceptibility results. However, chloramphenicol is also a good indicator when used with our recommended breakpoints. Although thiamphenicol is not approved for clinical use in the United States, it might be useful as a laboratory test agent to separate the CAT-producing organisms. Studies are presently being conducted in our laboratory to evaluate the usefulness of thiamphenicol as an alternative substrate in assaying for CAT.

The importance of correlating disk diffusion and MIC breakpoints with CAT production by resistant strains has been recognized by the NCCLS. In the next edition of the susceptibility standards for disk diffusion and MIC tests, the two methods used in this study to detect CAT production will be recommended for *H. influenzae*. The manufacturer of the disk used in this study for detection of CAT activity recommends it only for *H. influenzae*, but it can also be used to detect CAT activity in *S. pneumoniae*, even though the intensity of the color in the positive test is not as great as with *H. influenzae*. On the other hand, this test was consistently negative for chloramphenicol-resistant *Aerococcus* species.

In summary, our data show that common breakpoints (MIC and disk diffusion) and the CAT test can be used to detect chloramphenicol resistance in *H. influenzae* and *S. pneumoniae*. Different breakpoints are required to detect resistance in *Aerococcus* species; however, the CAT tests in their present form cannot be relied upon to indicate chloramphenicol resistance. Our data also show that CAT production is indicated better by the susceptibility results for thiamphenicol than the results for chloramphenicol.

**LITERATURE CITED**


