In Vitro Chloramphenicol Susceptibility Testing of *Haemophilus influenzae*: Disk Diffusion Procedures and Assays for Chloramphenicol Acetyltransferase

GARY V. DOERN,1,2* GARY S. DAUM,3 AND TRACEY A. TUBERT1

Department of Clinical Microbiology,1 Division of Infectious Disease,2 and Department of Pathology,3 University of Massachusetts Medical Center, Worcester, Massachusetts 01605

Received 24 February 1987/Accepted 7 May 1987

The activity of chloramphenicol against 100 different strains of *Haemophilus influenzae* was assessed by a macrotube broth dilution technique and by a standardized disk diffusion method using both enriched chocolate agar (CHOC) and Mueller-Hinton agar containing 1.0% hemoglobin and 1.0% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) supplement (CHOC-MHA). Filter disks containing 30 μg of chloramphenicol were used with the disk diffusion procedure. The following zone diameter interpretive criteria were defined: CHOC-MHA, ≤25 mm = susceptible and ≥26 mm = resistant; CHOC, ≤28 mm = susceptible and ≥29 mm = resistant. All of the *H. influenzae* strains examined were also characterized by using two rapid assays for chloramphenicol acetyltransferase (CAT) activity: a 1-h tube method (t-CAT) and a 30-min procedure which used commercially available reagent-impregnated disks (d-CAT). The t-CAT procedure was found to be significantly more accurate than the d-CAT procedure as a means for demonstrating production of CAT.

*Haemophilus influenzae* is well recognized as an important cause of a variety of serious life-threatening infections in humans. Because of the high prevalence of ampicillin resistance among clinical isolates of *H. influenzae* in the United States (3; G. V. Doern, Antimicrob. Newsl. 3:28–34, 1986), chloramphenicol is often used to treat systemic infections. Recently, however, resistance to this agent has been reported (5, 9). Indeed, in a large multicenter surveillance study conducted in 1984, 0.6% of clinical *H. influenzae* isolates were determined to be resistant to chloramphenicol (3). The mechanism of resistance among most, but not all, chloramphenicol-resistant strains is production of an enzyme, chloramphenicol acetyltransferase (CAT) (1, 2, 8).

Because of the potential for chloramphenicol resistance, the clinical laboratory is now faced with the necessity of being able to perform chloramphenicol susceptibility tests with *H. influenzae*. This can be accomplished using broth and agar dilution techniques (7), with a standardized disk diffusion procedure (6) or by determining the presence of CAT, recognizing that not all chloramphenicol-resistant strains necessarily produce this enzyme (1, 2). The intent of the present investigation was to examine the utility of a standardized disk diffusion procedure and two assays of CAT activity as means for assessing the chloramphenicol susceptibility of *H. influenzae*.

MATERIALS AND METHODS

Strains. A total of 100 strains of *H. influenzae* were examined. Fifty of these strains were obtained from patients in central Massachusetts with a variety of *H. influenzae* infections during the 6-year period 1979 to 1985. The remaining 50 strains were provided by J. H. Jorgensen, University of Texas Health Science Center at San Antonio, San Antonio, and represented clinical isolates obtained from several different locations in the United States, as well as from various western European countries. Before testing, stock cultures were inoculated onto enriched chocolate agar (CHOC) plates and incubated at 35°C in 5 to 7% CO₂ for 18 to 24 h. Isolated colonies from the initial plate were transferred to a second CHOC plate which was incubated under identical conditions. Growth from this plate was used to prepare inocula for all subsequent tests.

Chloramphenicol MIC determinations. Chloramphenicol MICs were determined by using a macrobroth tube dilution procedure in cation-supplemented Mueller-Hinton broth containing 3% lysed horse blood and 15 μg of NAD (Sigma Chemical Co., St. Louis, Mo.) per ml (pH 7.2; final volume, 2.0 ml). Chloramphenicol, obtained as reagent grade powder from Parke, Davis & Co., Morris Plains, N.J., was tested at twofold concentration increments from 0.008 to 128 μg/ml. The final concentration of the test organism was ca. 5 × 10⁵ CFU/ml. Tubes were incubated for 24 h at 35°C in 5 to 7% CO₂ and examined macroscopically for evidence of turbidity. The MIC was defined as the lowest concentration of chloramphenicol tested which yielded no macroscopic evidence of turbidity.

Disk diffusion susceptibility studies. Disk diffusion susceptibility tests were performed precisely as described by the National Committee for Clinical Laboratory Standards (NCCLS) (b). Two different agar media, both obtained preprepared in 100-mm plastic petri dishes from Scott Laboratories, Inc., Fiskeville, R.I., were used: CHOC GC agar base supplemented with 1% bovine hemoglobin and 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) and Mueller-Hinton agar containing 1% bovine hemoglobin and 1% IsoVitaleX (CHOC-MHA). Paper disks contained 30 μg of chloramphenicol (BBL). All determinations were made in duplicate without knowledge of the companion result.

CAT assays. A rapid tube test for CAT activity (t-CAT) was performed as described by Azumen et al. (1). A second test for CAT activity (d-CAT) was performed using commercially available reagent-impregnated paper disks (Remel, Lenexa, Kans.) in accordance with the instructions of the manufacturer. Both CAT tests were performed in duplicate without knowledge of the companion result.

* Corresponding author.

1453
Concentration, ug/ml

FIG. 1. Chloramphenicol MICs and zones of inhibition obtained with CHOC-MHA (A) and CHOC (B).

Linear regression analysis. Comparison of zones of inhibition obtained with the disk diffusion procedure with MICs (log$_2$) obtained with the macrotube broth dilution technique was accomplished by linear regression analysis using the method of least squares.

RESULTS

Comparison of the results of duplicate determinations of zones of inhibition obtained in the disk diffusion procedure with CHOC-MHA and CHOC revealed similar degrees of reproducibility. When CHOC-MHA was used as the test medium, differences of $\leq$4 mm between the results of duplicate determinations were observed with 96 of 100 test organisms. In two instances, the zones of inhibition varied by 5 mm, and in one instance each they varied by 6 and 7 mm. When determinations were made with CHOC as the test medium, differences of $\leq$4 mm were noted with 98 of the 100 test strains. One strain exhibited a variation of 5 mm; the results with another strain varied by 6 mm. The zones of inhibition were distinct and easily measured with both media.

The relationships between MICs and zones of inhibition for the 100 H. influenzae strains determined using CHOC-MHA and CHOC are shown in Fig. 1A and B, respectively. The averages of duplicate determinations of zones of inhibition were used. There was a bimodal distribution of organisms with respect to chloramphenicol MICs. For 16 strains, MICs were $\geq$8.0 $\mu$g/ml (8.0 $\mu$g/ml for 6 strains, 16.0 $\mu$g/ml for 9 strains, and 32.0 $\mu$g/ml for 1 strain). MICs for the 84 remaining strains were $\leq$1.0 $\mu$g/ml (1.0 $\mu$g/ml for 45 strains, 0.5 $\mu$g/ml for 38 strains, and 0.25 $\mu$g/ml for 1 strain).

When CHOC-MHA was used as the disk diffusion test medium (Fig. 1A), all 84 strains for which the chloramphenicol MICs were $\leq$1.0 $\mu$g/ml were found to have zones of inhibition $\geq$28 mm; each of the 16 strains for which the MICs were $\geq$8.0 $\mu$g/ml had zone sizes $\leq$22 mm. When CHOC was used (Fig. 1B), the zone size correlates were $\geq$31 and $\leq$25 mm, respectively. Correlation coefficients derived from linear regression analyses of the relationship between zones of inhibition and MICs (log$_2$) were 0.937 when CHOC-MHA was used and 0.917 when CHOC was used.

In assays for CAT activity, 14 of the 16 H. influenzae strains for which the chloramphenicol MICs were $\geq$8.0 $\mu$g/ml were determined to be positive with t-CAT procedure. The remaining two strains were t-CAT negative. The chloramphenicol MIC for both of these strains was 8.0 $\mu$g/ml. Their zones of inhibition on CHOC-MHA were 22 mm; on CHOC they produced zone sizes of 23 and 25 mm. All of the 84 strains for which the chloramphenicol MICs were $\leq$1.0 $\mu$g/ml were negative with the t-CAT assay. The results of duplicate t-CAT determinations were identical with all 100 strains.

When CAT activity was assessed with the d-CAT assay, 9 of the 16 strains for which the MICs were $\geq$8.0 $\mu$g/ml were determined to be unequivocally positive, i.e., the results of duplicate determinations were both interpreted as positive. Of these 16 strains, 3 yielded equivocal results with the d-CAT procedure, i.e., one of the duplicate determinations was judged positive, and the other was judged negative. The four remaining H. influenzae strains for which the chloramphenicol MICs were $\geq$8.0 $\mu$g/ml were unequivocally negative. Two of these strains were the ones that yielded conclusively negative results with the t-CAT procedure. Among 84 H. influenzae strains for which the chloramphenicol MICs were $\leq$1.0 $\mu$g/ml, the d-CAT test result was considered equivocal for 4 strains and conclusively negative for the remaining 80 strains.

In general, the distinctness of the color change representative of a positive reaction was much greater with the t-CAT assay than with the d-CAT procedure. Indeed, great difficulty was often encountered when interpreting d-CAT test results.

DISCUSSION

In vitro testing of H. influenzae susceptibility to chloramphenicol is important because resistance to this agent has been clearly documented (3, 5, 8, 9). Although the exact prevalence of chloramphenicol resistance is unknown, a large multicenter surveillance study which examined well over 3,000 clinical isolates of H. influenzae in 1984 revealed a 0.6% rate of resistance in the United States (3).

One method for assessing the in vitro activity of chloramphenicol against H. influenzae is a standardized disk diffusion susceptibility test. In the most recent approved standard for performance of disk susceptibility tests (NCCLS standard M2-A3), the use of CHOC-MHA is advocated for testing H. influenzae (6). The same chloramphenicol zone diameter interpretive criteria that are applied to nonfastidious bacteria (i.e., $\leq$12 mm = resistant and $\geq$18 mm = susceptible) are recommended for use with H. influenzae (6).

In the present investigation, use of the NCCLS guidelines for disk susceptibility testing of H. influenzae on CHOC-MHA revealed that 4 of 16 strains for which the chloramphenicol MICs were $\geq$8.0 $\mu$g/ml would have been categorized as susceptible since their zones of inhibition were $\geq$18 mm. Furthermore, none of the 12 remaining strains would have been categorized as resistant, since their zones of inhibition (13 to 17 mm) were above the NCCLS cutoff for resistance ($\leq$12 mm).

The significance of these observations is largely dependent on whether the 16 H. influenzae strains for which the MICs
were \( \geq 8.0 \mu g/ml \) are considered clinically resistant to chloramphenicol. For the following reasons, it is our contention that such strains should be considered chloramphenicol resistant, at least for systemic infections such as meningitis. When chloramphenicol is administered intravenously at a dose of 50 to 100 mg/kg per day, levels in plasma of 10 to 20 \( \mu g/ml \) are attained (4). Levels in the cerebrospinal fluid are approximately 50% of that in plasma (i.e., 5 to 10 \( \mu g/ml \)) (4). Therefore, meningitis caused by \( H. influenzae \) strains for which the MICs are \( \geq 8.0 \mu g/ml \) is unlikely to be treated effectively with chloramphenicol.

If this is so, the results of the present investigation indicate that the zone size interpretive criteria applied to \( H. influenzae \) when tested with 30-\( \mu g \) chloramphenicol disks on CHOC-MHA should be changed. Based on the results of this study, we recommend that \( H. influenzae \) strains with zones of inhibition \( \geq 26 \) mm be considered susceptible and those with zone sizes \( \leq 25 \) mm be viewed as resistant.

Because CHOC-MHA is a relatively specialized medium which may not always be available, particularly in small clinical microbiology laboratories, we also evaluated CHOC as a medium for performing chloramphenicol disk diffusion susceptibility tests with \( H. influenzae \). Although the zone size interpretive criteria would be different from those used with CHOC-MHA, CHOC was satisfactory. There was essentially no difference in the reproducibility of zone sizes on the two media. Furthermore, the correlation between MICs and zones of inhibition determined with CHOC (\( r = 0.917 \)) was comparable to that observed with CHOC-MHA (\( r = 0.937 \)). Finally, the same bimodal distribution was observed on both media. Based on the results of this investigation, we recommend that the following interpretive criteria be applied when testing \( H. influenzae \) on CHOC with 30-\( \mu g \) chloramphenicol disks: \( \geq 29 \) mm = susceptible and \( \leq 28 \) mm = resistant.

We do not advocate definition of an intermediate (or moderately susceptible) category for either CHOC-MHA or CHOC. The reasons for this are twofold. First, because of the distinct bimodal distribution of test strains in the present investigation, no organisms for which the MICs were intermediate were noted. In other words, organisms appeared to be either resistant or susceptible. Second, by establishing a single zone size cutoff in close proximity to that for the apparently susceptible strains (i.e., those for which the MICs are \( \leq 1.0 \mu g/ml \)), we mitigate against the most significant interpretive error, a false-susceptible result.

The results of this study also confirm the previous observation that not all chloramphenicol-resistant strains of \( H. influenzae \) produce CAT, at least as detectable by a rapid tube assay (1, 2). This assay has previously been shown to yield results equivalent to those obtained with a standard spectrophotometric method (1). Of 16 \( H. influenzae \) strains examined in this study which were apparently resistant to chloramphenicol, 2 produced negative results with the t-CAT procedure. However, this should not be construed as indicating that 12.5% of all chloramphenicol-resistant \( H. influenzae \) strains lack CAT. The organisms examined were selected strains of \( H. influenzae \); therefore no judgments pertaining to prevalence can be made. These results do indicate, however, that in vitro CAT assays cannot be used as a sole criterion for chloramphenicol susceptibility among clinical isolates of \( H. influenzae \). Furthermore, a commercially available disk test should not be used for in vitro screening of CAT activity. This procedure yielded numerous false-negative and equivocal results and was difficult to interpret.

In conclusion, we offer the following suggestions regarding in vitro susceptibility testing of clinically significant isolates of \( H. influenzae \). Upon recovery of the organism in pure culture, the rapid tube test for CAT activity may be used and, if positive, the organism can be considered resistant to chloramphenicol. This procedure requires approximately 70 min. If a negative result is obtained, a direct test of chloramphenicol activity should be performed. This may consist of a standardized disk diffusion susceptibility test using either CHOC-MHA or CHOC as the medium. The optimum zone diameter interpretive criteria are those delineated above.

ACKNOWLEDGMENT

We acknowledge the excellent secretarial assistance of Karen Spiewak.

LITERATURE CITED

ERRATA

In Vitro Chloramphenicol Susceptibility Testing of Haemophilus influenzae: Disk Diffusion Procedures and Assays for Chloramphenicol Acetyltransferase

GARY V. DOERN, GARY S. DAUM, AND TRACEY A. TUBERT

Department of Clinical Microbiology, Division of Infectious Disease, and Department of Pathology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

Volume 25, no. 8, p. 1453, abstract, line 6. The zone diameter interpretive criteria should be as follows: “CHOC-MHA, \( \leq 25 \) mm = resistant and \( \geq 26 \) mm = susceptible; CHOC, \( \leq 28 \) mm = resistant and \( \geq 29 \) mm = susceptible.”

Failure of Multiple Passages To Increase Chlamydial Recovery

JULIUS SCHACHTER AND DAVID H. MARTIN

Department of Laboratory Medicine, University of California, San Francisco, California 94143, and Department of Medicine, Louisiana State University Medical Center, New Orleans, Louisiana 70112-2822

Volume 25, no. 10, p. 1851. The last two lines on the page were inadvertently cut off. They should read as follows: “...SF, loosely capped vials were incubated in 5% CO\(_2\). Blind passage was performed by disrupting the monolayers on a...”