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Influence of Variations in Test Methods on Susceptibility of *Haemophilus influenzae* to Ampicillin, Azithromycin, Clarithromycin, and Telithromycin

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The National Committee for Clinical Laboratory Standards standard broth microdilution method for testing the susceptibility of *Haemophilus influenzae* to ampicillin, azithromycin, clarithromycin, and telithromycin was evaluated by altering one variable at a time. Variables that were tested included age of colony for inoculum preparation, inoculum density, test medium, incubation atmosphere, and incubation time. For the macrolide, azalide, and ketolide agents, incubation in 5 to 7% CO_2 most significantly affected the MICs, producing nearly twofold increases for clarithromycin and telithromycin and a greater than threefold increase for azithromycin. For ampicillin, a 10-fold increase in inoculum density increased the geometric mean MICs for β-lactamase-negative strains from 1.50 to 2.45 μg/ml. In addition, 206 *H. influenzae* strains were tested for their susceptibilities to the same drugs by the broth microdilution tests in two media, as well as by agar dilution tests, disk diffusion tests, and Etests, on six different agar media. The three standard methods with *Haemophilus* test medium (HTM) compared favorably with each other except for a high minor discrepancy rate (27%) by the disk diffusion test with ampicillin and clarithromycin. Agar dilution test MICs on the five comparative media were generally higher than those on HTM agar but were only rarely more than one twofold concentration higher. Etest MICs of azithromycin and telithromycin were more than twofold higher than agar dilution and broth microdilution MICs on HTM; ampicillin Etest MICs were nearly twofold lower. The use of media other than HTM agar appears to have a minimal effect on susceptibility test results for the ketolide, azalide, or macrolide drugs that we tested against *H. influenzae*.

The importance of standardization of antimicrobial susceptibility tests for *Haemophilus influenzae* is widely recognized (16). Standardized procedures for susceptibility testing of this species by dilution and disk diffusion (DD) methods have been promulgated by the National Committee for Clinical Laboratory Standards (NCCLS) for many years (11–15). The interpretive criteria published by NCCLS are applicable only if the NCCLS methods are precisely followed or if procedural modifications have been demonstrated to produce equivalent results (4). The latter appear to be rarely done, or are at least rarely published.

Many methods for susceptibility testing of *H. influenzae* have been used worldwide. Published quality assessment studies have shown significant discrepancies between laboratories, particularly when testing antibiotic-resistant isolates (19, 20). In the case of DD tests, such discrepancies have been attributed to variations in methods and the use of different interpretive criteria (19, 20). Procedural variations have included differences in media, supplements, disk content, and inoculum (20). Under controlled conditions, however, the DD test has shown good interlaboratory reproducibility (5).

The susceptibility of *H. influenzae* to macrolides has varied in different reports. The ketolide telithromycin (HMR 3647) has been reported to have in vitro activity against *H. influenzae* (1, 9; M. Otsuki, H. Harada, and T. Nishino, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-137, 1998; K. E. Piper, M. S. Rouse, R. Patel, W. R. Wilson, and J. M. Steckelberg, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. B-41, 1998), but the MICs at which 90% of isolates are inhibited range from 0.5 to 4.0 μg/ml in different investigations (Andre Bryskier, personal communication). Such wide ranges might reflect the differences in the susceptibilities of different populations of *H. influenzae*. On the other hand, differences in susceptibility test methods by different investigators might also account, at least in part, for such diverse results.

The present study was designed to determine the effects of deviations from the NCCLS methods for testing of the susceptibility of *H. influenzae* to four antimicrobial agents. The agents tested were azithromycin, clarithromycin, and telithromycin because of their relative newness and potential use against *H. influenzae* (1, 7, 8) and ampicillin because of its central role in susceptibility testing of this organism.

MATERIALS AND METHODS

The study was divided into two phases. In phase 1, 12 strains of *H. influenzae* were tested for their susceptibilities to the four drugs by the broth microdilution (BMD) method under various conditions. The variables examined included medium, incubation atmosphere, incubation time, inoculum density, and age of the colonies from which the inoculum was prepared. In phase 2, 206 isolates of *H. influenzae* were tested for their susceptibilities to the four antibiotics by the BMD, agar dilution (AD), DD, and Etest methods with a variety of test media that were used in studies that have been published over the past decade.

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TABLE 1. Geometric mean MICs of four antibiotics for 12 strains of *H. influenzae* tested under standard conditions compared to MICs with a single deviation from MICs obtained under standard conditions

Antimicrobial agent	Geometric mean MIC (μg/ml) under the following conditions:										
	Standard[a]	LHB medium	Atmosphere 5–7% CO_2	Inoculum		Incubation time		Age of colony used for inoculum			
				5×10^6 CFU/ml	5×10^4 CFU/ml	16 h	24 h	8 h	12 h	20 h	24 h
Telithromycin	2.00	1.68	3.78	2.12	1.50	2.00	2.12	2.00	2.25	2.38	2.00
Clarithromycin	7.55	5.99	12.7	9.51	5.66	7.55	8.48	8.48	8.98	8.98	8.98
Azithromycin	0.94	0.75	3.00	1.12	0.89	0.89	1.19	1.19	1.26	1.26	1.19
Ampicillin	1.50	1.33	1.33	2.45	1.19	1.50	1.41	1.59	1.41	1.19	1.41

[a] Sixteen-hour colonies suspended in saline and adjusted to yield an inoculum with ca. 5×10^5 CFU/ml in HTM broth, incubated for 20 h in ambient air.

Microorganisms. In phase 1, 12 strains of *H. influenzae* were tested and included 5 β-lactamase-negative, ampicillin-resistant (BLNAR) stock isolates, 5 fresh β-lactamase-negative, ampicillin-susceptible (BLNAS) strains (no more than four subcultures from the patient), and 2 *H. influenzae* quality control strains (strains ATCC 49247 and ATCC 49766). The 206 *H. influenzae* strains tested in phase 2 included 159 stock strains and 47 fresh clinical isolates. Of the 206 isolates, 61 were β-lactamase positive, 46 were BLNAR (MICs, ≥4.0 μg/ml), 13 were β-lactamase negative, ampicillin intermediate (MICs, 2.0 μg/ml), and 86 were BLNAS (MICs, ≤1.0 μg/ml). The two quality control strains listed above were tested on each day of testing.

Antimicrobial agents. Telithromycin was provided as a standardized powder by Hoechst Marion Roussel R & D, Romainville, France; clarithromycin, azithromycin, and ampicillin were procured from their respective U.S. manufacturers or other commercial sources. The following commercially prepared disks were used for DD tests: ampicillin, 10 μg; azithromycin, 15 μg; clarithromycin, 15 μg; and telithromycin, 15 μg. Etest strips were purchased from AB Biodisk.

Susceptibility Test Methods. (i) Phase 1. Each isolate was tested by the standardized BMD method with strict adherence to the procedures outlined by NCCLS (15). In addition, each was tested with the following modifications of that method alone and in combination: (i) age of colonies from which the inoculum was prepared, 8, 12, 16 (standard), 20, and 24 h; (ii) inoculum concentration, ca. 5×10^6, ca. 5×10^5 (standard), and ca. 5×10^4 CFU/ml (the two nonstandard concentrations were prepared from 16-h colonies only); (iii) test medium, *Haemophilus* test medium (HTM) (standard) and Mueller-Hinton broth supplemented with ca. 3% lysed horse blood and 20 μg of NAD per ml (LHB-3); (iv) incubation atmosphere, ambient air (standard) and 5 to 7% CO_2; and (v) incubation time, 16, 20 (standard), and 24 h. This combination of tests resulted in a total of 84 MIC results for each organism-antibiotic combination. The drugs were dispensed in trays as serial twofold concentrations ranging from 0.015 to 64 μg/ml for telithromycin, 0.015 to 16 μg/ml for azithromycin and ampicillin, and 0.03 to 64 μg/ml for clarithromycin and were stored at −70°C until the day of use.

(ii) Phase 2. BMD, AD, and DD tests were performed by the methods outlined by NCCLS (14, 15). Etest MICs were determined by the method recommended by the manufacturer and were rounded up to the nearest even $\log_2$ dilution interval. In addition to the standard HTM, BMD tests were also performed in LHB-3. AD tests, DD tests, and Etests were performed on HTM agar (standard), Mueller-Hinton agar (MHA) with 5% lysed horse blood and 20 μg of NAD per ml (LHB-5), MHA supplemented with 5% whole horse blood and 20 μg of NAD per ml (WHB), MHA supplemented with 1% hemoglobin and 1% IsoVitaleX (HGB), MHA supplemented with 3% Fildes peptic digest of blood (FIL), and Iso-Sensitest agar with 5% horse blood and 20 μg of NAD per ml (ISO). These tests were all incubated in 5 to 7% CO_2. The 47 fresh clinical isolates were also tested by the Etest in ambient air.

RESULTS

Phase 1. The geometric mean MICs of 12 tests with a single deviation from the standard method and the geometric MICs obtained by the standard method are compared in Table 1.

(i) Medium. Of the 48 tests in which LHB-3 was the only deviation from the standard HTM broth, all but one yielded MICs within 1 dilution of the MIC obtained by the standard test. There was, however, a slight skewing toward lower MICs of all drugs with tests in LHB-3. There were 504 tests in LHB-3 in which one or more additional test parameters were also varied, and 99.6% of these test results were within 1 $\log_2$ concentration of the results of the same tests in HTM broth.

(ii) CO_2. Incubation in 5 to 7% CO_2 as the sole variable resulted in nearly twofold increases in the clarithromycin and telithromycin MICs but a greater than threefold increase in the azithromycin MIC. Only 42% of all azithromycin MICs in CO_2 were within a 1 dilution interval from the MICs obtained by the standard method. That compares with 87% for telithromycin and 89% for clarithromycin. Ampicillin MICs were not affected by increased levels of CO_2.

(iii) Inoculum size. The mean colony count for the standard inoculum was 5.6×10^5 CFU/ml (range, 2.5×10^5 to 9.4×10^5 CFU/ml), that for the high inoculum targeted for 10 times the standard inoculum was 5.2×10^6 CFU/ml (range, 2.4×10^6 to 7.8×10^6 CFU/ml), and that for the low inoculum targeted for 1/10 the standard inoculum was 5.6×10^4 CFU/ml (range, 1.8×10^4 to 7.4×10^4 CFU/ml). There was minimal skewing toward lower MICs with the low inoculum and toward higher MICs with the high inoculum when the three macrolides were tested. Although the low inoculum had little effect on the ampicillin MICs, the high inoculum significantly increased the MICs for some strains. For 18% of these β-lactamase-nonproducing strains, the ampicillin MICs were increased fourfold with the denser inoculum. When the high or low inoculum was combined with changes to one or more other variables, ≥98.8% of the results were within 1 dilution of the results obtained by the standard method except for ampicillin and the high inoculum (82.0%). The heavy inoculum moved the geometric mean MIC of ampicillin from the susceptible category to the intermediate category.

(iv) Incubation time. As a sole variable, incubation times of 16 and 24 h had no appreciable effect on the MICs. However, when combined with changes to other variables, there was a very slight skewing toward lower MICs with 16 h of incubation and higher MICs with 24 h of incubation. Over 99.5% of macrolide MICs were within 1 dilution of the results obtained by the standard method, but only 95.8 and 95.2% of the ampicillin MICs at 16 and 24 h, respectively, fell in this range.

(v) Age of colonies used for inoculum preparation. In tests conducted to determine the effects of inocula prepared from colonies that varied in age from 8 to 24 h on otherwise standardized tests, only minor variations in MICs were noted, but no trends or consistent patterns were observed.

Phase 2. The geometric mean MICs generated with different media and by different methods are compared in Table 2.

TABLE 2. Geometric mean MICs by three methods and mean zone diameters for four antibiotics on multiple media against 206 strains of *H. influenzae*

Antimicrobial agent	Geometric mean MIC (μg/ml) under the following conditions:														Mean zone diam (mm) by DD method (CO_2)					
	BMD[a] (air)		AD method (CO_2)						Etest (CO_2)											
	HTM	LHB-3	HTM	LHB-5	WHB	HGB	FIL	ISO	HTM	LHB-5	WHB	HGB	FIL	ISO	HTM	LHB-5	WHB	HGB	FIL	ISO
Telithromycin	1.87	1.69	1.34	2.44	2.29	3.02	1.92	2.00	4.06	4.60	4.49	5.91	3.65	3.82	19.6	19.2	19.2	17.8	20.7	20.4
Clarithromycin	8.61	7.13	5.35	8.54	8.02	8.07	6.22	7.05	8.50	10.3	9.72	10.3	7.61	8.52	16.5	16.0	16.2	16.0	17.7	17.2
Azithromycin	1.23	1.17	1.49	1.93	1.98	2.01	1.34	1.48	3.86	6.67	3.70	4.11	2.83	2.96	20.1	20.9	20.5	20.9	22.6	21.8
Ampicillin	2.49	2.55	1.91	2.42	3.00	3.60	2.57	2.23	1.23	1.62	1.53	1.65	1.26	1.64	21.6	20.8	20.5	20.4	21.8	21.1

[a] BMD tests in two broth media (Table 1).

(i) Medium Comparisons. By the BMD method only two media were compared: HTM and LHB-3. There was minimal skewing toward lower MICs when tests were performed on LHB-3 for clarithromycin and telithromycin, as seen in Phase 1, but MICs essentially comparable to those obtained by the standard method were obtained for the other two drugs. With ampicillin, 96.1% of the results were within 1 dilution of each other; for the other 3 drugs, 99% of the results were within 1 dilution of each other.

For the AD test method, the MICs obtained on HTM agar were compared with those obtained on each of five other agar media. With all five media there was skewing toward higher MICs compared to those obtained on HTM. The percentages of MIC results on these media that were within 1 dilution of those on HTM ranged from 74% on HGB to 90% on ISO for telithromycin, 87% on HGB to 90% on FIL for clarithromycin, 62% on HGB to 84% on ISO for ampicillin, and 91% on WHB to 97% on FIL for azithromycin. Over 95% of the MICs that differed by more than 1 dilution from the MICs obtained on HTM were on the high side. With ampicillin, β-lactamase-positive strains had a disproportionately greater number of results outside the ±1 dilution range of the results obtained by the standard method, but all strains would have been classified as resistant.

The Etest MICs were more comparable to each other on the six different agar media. Ampicillin performed the poorest, with a range of 90% of the MICs obtained on HGB to 95% of the MICs obtained on FIL being within 1 dilution of the MICs obtained on HTM. For the other three drugs, 95% of the MICs obtained on all five media were within 1 dilution of the MICs obtained on HTM.

The overall mean zone diameters on LHB, WHB, HGB, and ISO were within 1 mm of those on HTM around telithromycin, clarithromycin, and azithromycin disks, with the exception of azithromycin on ISO, which was 1.7 mm larger. On FIL the mean zone diameters were 1.1 to 2.5 mm larger than those on HTM. Mean zone diameter variations for ampicillin ranged from 0.2 mm larger (FIL) to 1.2 mm smaller (HGB) compared to those on HTM. Individual strains showed greater differences between agar media. The percentages of isolates with ampicillin zone diameters on the five test media that differed by >3 mm from those on HTM were 7.8% on WHB, 10.0% on LHB-5, 11.8% on HGB, 19.8% on ISO, and 21.1% on FIL.

(ii) Method comparison. Even though AD tests were incubated in the presence of increased levels of CO_2 and BMD tests were incubated in air, AD and BMD test results compared reasonably well with each other when the tests were performed on the same medium. When tested on HTM, the geometric mean MICs obtained by the two methods were within 0.5 $\log_2$ concentration of each other. Despite this, significant minor interpretive discrepancies were observed with clarithromycin when susceptibility categories were compared by using the breakpoints listed by NCCLS (15). The minor discrepancy rates were 25.7 and 22.8% when tests were performed on HTM and LHB, respectively. This is undoubtedly due to the fact that over 80% of the clarithromycin MICs were within 1 dilution of the intermediate MIC of 16 μg/ml.

Etest clarithromycin MICs correlated well with both AD and BMD test MICs (Table 2), but the same high minor interpretive discrepancy rate was observed. For the other three drugs there were poorer correlations of the MICs obtained by the BMD test or the AD test. The telithromycin and azithromycin MICs obtained by the Etest were two to three times higher than those obtained by the BMD and AD tests, and the ampicillin MICs obtained by the Etest were approximately one-half those obtained by the AD and BMD tests. The major discrepancy rates for azithromycin were 32.5 and 27.7% when it was tested on HTM and LHB-5, respectively.

DD test categorical discrepancy rates were calculated for the three drugs for which NCCLS breakpoints are provided (14). Azithromycin DD tests performed very well compared to the BMD and AD tests on all media, with no more than one discrepancy on each medium. With clarithromycin there was a relatively high minor discrepancy rate (16.5 to 27.2%), again due to the high proportion of results clustering around the MIC for intermediate resistance. With ampicillin there was a high very major discrepancy rate (up to 4.4%) as well as a high minor discrepancy rate (up to 27.2%). The great majority of these discrepancies were due to BLNAR strains.

(iii) Quality control. All MICs obtained by the standard method and zone diameters obtained by the DD test with the BLNAR control strain of *H. influenzae* (strain ATCC 49247) for the three drugs for which ranges have been published by NCCLS were within the appropriate ranges. The BMD trays, stored at −70°C, were used over a 4-month period, and the mode (range) MICs on HTM for this organism were as follows: ampicillin, 4.0 μg/ml (4.0 to 8.0 μg/ml); azithromycin, 2.0 μg/ml (1.0 to 4.0 μg/ml); and clarithromycin, 8.0/16 μg/ml (4.0 to 16 μg/ml). For the BLNAS control strain of *H. influenzae* (strain ATCC 49766), the corresponding values were as follows: ampicillin, 0.25 μg/ml (0.25 to 0.25 μg/ml); azithromycin, 4.0 μg/ml (2.0 to 4.0 μg/ml); and clarithromycin, 32 μg/ml (16 to 32 μg/ml). For both organisms there was no trend toward

increasing or decreasing MICs over time, indicating no significant deterioration of the drugs or the medium.

DISCUSSION

When testing a macrolide, an azalide, and a ketolide, the only variable in test conditions that resulted in consistently different results was the incubation of BMD tests in 5 to 7% CO_2 (Table 1). This was not unexpected since CO_2 lowers the pH of the medium, and it has been well documented that decreased pH decreases the activities of these compounds (3, 6, 17). When tested by the BMD method in air, the MICs of these three drugs were within 0.5 $\log_2$ concentration of those obtained by the AD test incubated in 5 to 7% CO_2, which are the standard atmospheres for these two procedures (15). The use of LHB-3 resulted in slight skewing toward lower MICs compared to those obtained by tests on HTM, as has been reported previously (2, 10, 18). The other variables that we studied produced only slight skewing of the results, and the geometric mean MICs remained within 0.5 $\log_2$ concentration of that obtained by the standard NCCLS method.

The MICs of these three drugs determined by the Etest were consistently higher than those determined by the BMD and AD tests, particularly with azithromycin and telithromycin, the MICs of which were twofold higher (Table 2). This may be related, at least in part, to the fact that agar media are incubated in CO_2, whereas the BMD tests are performed in ambient air (3). The increased levels of CO_2 in the atmosphere does not appear to be a factor when the MICs obtained by the BMD test were compared with the MICs obtained by the AD test. Incubation in CO_2 also provides smaller zones of inhibition (3), but the interpretive criteria for the DD test are based on incubation of the standard DD test in the presence of increased levels of CO_2 and incubation of the standard BMD test in ambient air. The 47 fresh clinical isolates that were included in phase 2 were also tested by the Etest in air, and the MICs were significantly lower than those obtained in the presence of CO_2 (data not shown): 3 of the 47 (6%) strains failed to grow on the agar medium in air. Thus, it appears that the Etest will perform comparably to other methods if it is incubated in air, but some strains will not grow on HTM agar.

From these data, we conclude that major differences between the MICs at which 90% of isolates are inhibited reported by different investigators might reflect different study populations of *H. influenzae* and cannot be accounted for solely by differences in test methods. It will be interesting to learn whether telithromycin proves to be clinically effective in the treatment of *H. influenzae* infections.

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