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Problems with Current Recommendations for Susceptibility Testing of *Haemophilus influenzae*

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We compared results of MIC and disk susceptibility tests on *Haemophilus* test medium (HTM) and those on comparative media. Ampicillin MICs were determined with seven ampicillin-resistant, non-β-lactamase-producing (Amp^{r}NBLP) isolates by using HTM and supplemented brain heart infusion (sBHI) agar. Ampicillin and amoxicillin-clavulanate disk tests with 16 Amp^{r}NBLP strains, 18 ampicillin-susceptible (Amp^s) isolates, and 17 ampicillin-resistant, β-lactamase-producing (Amp^{r}BLP) strains were performed by using five media: laboratory-prepared HTM (PHTM), commercial HTM (CHTM), sBHI, enriched chocolate agar, and Mueller-Hinton chocolate agar. We observed that five of seven and three of seven Amp^{r}NBLP strains were misclassified as susceptible with PHTM (MIC, <2 μg/ml) with inocula of 10^3 and 10^5 CFU, respectively, but were resistant with sBHI (MIC, ≥2 μg/ml). Whereas Mueller-Hinton chocolate agar and enriched chocolate agar plates supported the growth of all 51 strains by the disk tests, 37% (19 of 51) and 8% (4 of 51) of strains did not grow on PHTM and CHTM, respectively. Lack of growth on PHTM was observed for all three phenotypes; 7 of 18 Amp^s, 4 of 17 Amp^{r}BLP, and 8 of 16 Amp^{r}NBLP strains did not grow. The four strains that did not grow on CHTM were all Amp^{r}NBLP isolates. Zone sizes were significantly larger on PHTM than on the other media. Of the strains that were evaluable by the new National Committee for Clinical Laboratory Standards guidelines with either PHTM or CHTM, all Amp^s strains were classified as susceptible. Among the Amp^{r}BLP strains, CHTM correctly identified all as resistant, whereas PHTM detected two isolates to be intermediate. Among the Amp^{r}NBLP strains, CHTM and PHTM misclassified four (33%) and five (62%) isolates, respectively, as susceptible; an additional isolate was identified as intermediate on both media. We conclude that there is strain-dependent growth on HTM, that adoption of this medium for routine *Haemophilus* susceptibility testing is problematic due to this growth variability, and that detection of Amp^{r}NBLP isolates would be unreliable.

In 1988, the National Committee for Clinical Laboratory Standards (NCCLS) recommended the use of *Haemophilus* test medium (HTM) for susceptibility testing of *Haemophilus influenzae* isolates in its M2-T4 publication (9); criteria for ampicillin disk susceptibility tests were modified to include an intermediate category (zone diameter, 22 to 24 mm) and larger zones for susceptibility (≥25 mm) and resistance (≤21 mm) (1, 9). Presumably, these changes would allow identification of all ampicillin-resistant strains by using the standard 10-μg ampicillin disk test. Previously, we showed with the former NCCLS criteria (7) and media that the 10-μg disk test, in contrast to the 2-μg ampicillin disk test, failed to detect 44% (8 of 18) of ampicillin-resistant, non-β-lactamase-producing (Amp^{r}NBLP) isolates; these isolates have an intermediate level of resistance compared with β-lactamase-producing strains (median ampicillin MIC, 8 versus 32 μg/ml, respectively, with an inoculum of 10^5 CFU) (4).

Recently, we used HTM to compare the in vitro susceptibility of *H. influenzae* to three cephem antibiotics (6). In that study, we observed that the ampicillin MICs with HTM were lower for Amp^{r}NBLP strains compared with our previous data obtained with the same isolates in our laboratory when we used supplemented brain heart infusion medium (sBHI; Difco Laboratories, Detroit, Mich.); those data prompted this investigation. We compared the ampicillin MICs using HTM (2) and sBHI agar with seven Amp^{r}NBLP strains, four control strains, two ampicillin-susceptible (Amp^s) strains, and two ampicillin-resistant, β-lactamase-producing (Amp^{r}BLP) isolates. In addition, we compared the ampicillin and amoxicillin-clavulanate disk susceptibilities of 18 Amp^s, 17 Amp^{r}BLP, and 16 Amp^{r}NBLP strains using five different media: (i) laboratory-prepared HTM (PHTM), (ii) commercially prepared HTM (CHTM), (iii) sBHI, (iv) enriched chocolate agar (CAE) medium, and (v) Mueller-Hinton chocolate agar (MHCA) medium.

MATERIALS AND METHODS

Strains. Consecutive clinical strains with the Amp^s and Amp^{r}BLP phenotypes isolated from 1988 to 1989 from children at Children's Hospital and Medical Center, Seattle, Wash., were chosen for study. Isolates were tested for β-lactamase production by use of nitrocefin-impregnated paper disks (Cefinase; BBL Microbiology Systems, Cockeysville, Md.) and were examined for agglutination with serotype b and polyvalent antisera (Difco). All strains had an obligatory requirement for β-NAD$^+$ (V factor) and hemin (X factor) for growth when incubated at 37°C. Strains were biotyped as I through VIII based on the production of urease, ornithine decarboxylase, and indole (3). Of the 18 Amp^s isolates, 10 were serotype b (7 were biotype I and 3 were biotype II), and 5 were isolated from blood and 5 were isolated from cerebrospinal fluid. Eight strains were nontypeable and of the following biotypes: biotype I, one strain; biotype II, three strains; biotype III, three strains; and biotype IV, one strain. Four of these were respiratory

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TABLE 1. MICs for *H. influenzae* isolates

Phenotype (no. of isolates)	Ampicillin MIC (μg/ml) for individual isolates[a]			
	sBHI		PHTM	
	10^3 CFU	10^5 CFU	10^3 CFU	10^5 CFU
Amp^s (2)	0.5	0.5	0.25	0.25
Amp^{r}BLP (2)	8	32, >32	4	32, >32
Amp^{r}NBLP (7)	$2, 4_4, 8_2$	4_4, 8, 16, >32	0.125_2, 0.5, 1.5_2, 4_2	$0.25, 1_2, 8_2$, 16, >32

[a] The agar dilution technique was used with PHTM or sBHI medium as described in the text. The subscript indicates the number of isolates with the indicated MIC.

isolates (sputum, trachea, or lung), two were eye isolates, and two were control strains (strain MAP [a laboratory strain] and ATCC 33391). Of the 17 Amp^{r}BLP strains, 10 were serotype b (9 were biotype I and 1 was biotype II), 5 were isolated from cerebrospinal fluid, 4 were isolated from blood, and 1 was isolated from lung. Seven isolates were nontypeable and of the following biotypes: biotype I, two strains; biotype II, one strain; biotype III, one strain; biotype IV, one strain; biotype V, two strains. Four strains were respiratory isolates, one strain was from blood, one strain was from ear, and one was control strain ATCC 33929. Of the 16 Amp^{r}NBLP strains, all were nontypeable and of the following biotypes: biotype I, four strains; biotype II, three strains; biotype III, three strains; biotype IV, four strains; biotype V, one strain; biotype VIII, one strain. Thirteen strains were respiratory isolates, two were isolated from blood, and one was an eye isolate. Four isolates were isolated in New Zealand, four were isolated in Boston, Mass., four were from the United Kingdom, three were from The Netherlands, and one was from Seattle.

Media. The media used in this study included MHCA and CAE, which were supplied by Prepared Media Laboratories (Tualatin, Oreg.), and HTM, which was prepared in our laboratory (PHTM) as originally described (2) by using Mueller-Hinton base and yeast extract from Difco Laboratories and hematin, which was from Sigma Chemical Co. (St. Louis, Mo.). The hematin for PHTM was prepared fresh each time the medium was made and was dissolved in NaOH as described previously (2) with constant stirring with a magnetic stir bar on a heated hot plate; the hematin suspension in an Erlenmeyer flask with the stir bar was placed in a beaker of boiling H_2O on a hot plate and stirred until it was fully dissolved, and a final concentration of 15 μg of hematin per ml and 5 mg of yeast extract per ml were added prior to autoclaving. After autoclaving, 15 μg of filter-sterilized β-NAD (Sigma) per ml was added aseptically to each liter of sterile cooled medium just before medium was poured into the plates. PHTM plates were used within 5 days of preparation. CHTM was obtained from Remel Laboratories (Lenexa, Kans.). Brain heart infusion agar (Difco) was supplemented with 15 μg of β-NAD (V factor) per ml, 15 μg of L-histidine per ml, and 15 μg of hemin chloride (X factor) per ml. Cultures were incubated at 37°C in 5% CO_2.

Susceptibility testing. (i) Agar dilution MIC. Seven AMP^{r}NBLP strains, two Amp^s strains (including ATCC 33391), and two Amp^{r}BLP strains (including ATCC 33929) were tested in duplicate with ampicillin. The strains were inoculated onto fresh chocolate agar plates from vials of skim milk that were stored at −70°C and were incubated overnight in 5% CO_2 at 37°C. Cells were harvested after overnight growth and diluted in phosphate-buffered saline to give final inocula of 10^3 and 10^5 CFU when they were plated with a Steers replicator on PHTM and sBHI agar containing graded antibiotic concentrations. Both media were plated at the same time in duplicate. The MIC was defined as the lowest concentration of antibiotic which inhibited visible growth of the inoculum in comparison with growth on antibiotic-free medium. Plates were examined after 18 to 24 h of incubation in 5% CO_2 at 37°C. The ampicillin concentrations tested included 1.5 μg/ml and at twofold dilutions from 0.125 to 32 μg/ml.

(ii) Disk susceptibility tests. Strains that were stored in skim milk at −70°C were used after overnight growth on chocolate agar plates. Inocula were prepared visually to match the turbidity of a 0.5 McFarland standard, and the disk diffusion tests were performed by the guidelines of the NCCLS M2-T4 publication (9). Antimicrobial disks containing 2 and 10 μg of ampicillin and a third disk containing 10 μg of amoxicillin and 20 μg of clavulanate were placed on the surfaces of plates (diameter, 100 mm); the agar depth was 4 mm, and two or three disks were placed on each plate. Four media, MHCA, CAE, PHTM, and sBHI, were tested at the same time in duplicate, whereas CHTM was tested later. Plates were incubated for 18 to 24 h before measurements were made by three different readers; duplicate values were averaged and rounded to the nearest millimeter. Unless two of the three values made by the readers were identical, the median value of the three was taken as the definitive zone size for that medium. If growth was insufficient to interpret a zone diameter; that is, if it was spotty, sparse, or light or if no growth was observed, single disks were placed on individual plates and a strain-inoculated plate with no disk was used as a control.

Statistical analysis. The ability of HTM to support growth was compared with the abilities of the other media to support growth for each of the phenotypic groups by using the Fisher exact test. Zone diameters for each of the three disk tests on individual media were compared by using a paired *t* test.

RESULTS

Agar dilution susceptibility. Table 1 shows the ampicillin MICs for representatives of the three phenotypic groups tested. No overlap was observed between Amp^s and

TABLE 2. Growth of *H. influenzae* on test media

Phenotype	No. of isolates[a]									
	sBHI		PHTM		CHTM		MHCA		CAE	
	G	NG	G	NG	G	NG	G	NG	G	NG
Amp^s	18	0	11	7	18	0	18	0	18	0
Amp^{r}BLP	17	0	13	4	17	0	17	0	17	0
Amp^{r}NBLP	15	1	8	8	12	4	16	0	16	0

[a] G, Growth; NG, no growth.

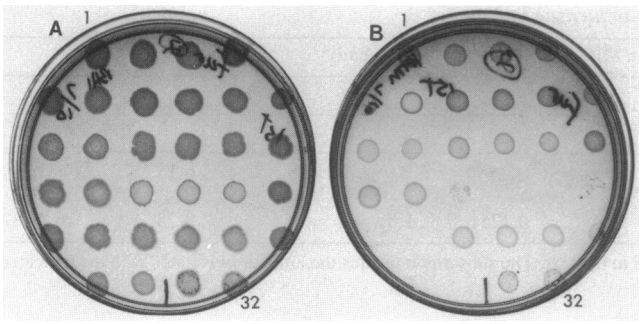

FIG. 1. Differences in growth of *H. influenzae* on sBHI (A) and PHTM (B). A total of 32 spots were inoculated with a Steers replicator and were numbered from 1 to 32 from left to right. A total of 11 strains are represented with three different inocula (10^5, 10^4, and 10^3 CFU) except for the last strain (spots 31 and 32) which had inocula of only 10^5 and 10^4, respectively. Amp^{r}BLP strains are present in spots 1 to 3, 13 to 15, 22 to 24, 28 to 30, and 31 and 32. Amp^s isolates were inoculated onto spots 4 to 6, 19 to 21, and 25 to 27. Amp^{r}NBLP strains are represented in spots 7 to 9, 10 to 12, and 16 to 18.

Amp^{r}NBLP strains with sBHI at either inoculum tested, whereas three of seven Amp^{r}NBLP strains were incorrectly classified as susceptible (MIC, $\leq$0.5 μg/ml) with PHTM at an inoculum of 10^3 CFU. If resistance is defined as an ampicillin MIC of $\geq$2 μg/ml, then five of seven and three of seven resistant strains were incorrectly classified as susceptible with PHTM at inocula of 10^3 and 10^5 CFU, respectively (Table 1). In contrast, all seven strains appeared to be resistant with sBHI with both inocula tested. Although HTM is a clear medium, determination of MIC endpoints often required removal of the lid and the use of tangential light to detect growth.

Growth on different media. Figure 1 shows differences in the growth of *H. influenzae* on control plates, that is, with no added antibiotic, from an agar dilution MIC test with strains replicated during the same experiment onto sBHI or PHTM agar. MHCA and CAE media supported the growth of all 51 isolates, and sBHI failed to support the growth of 1 Amp^{r}NBLP isolate (Table 2). In contrast, PHTM failed to support the growth of 19 of the 51 strains (37%; $P < 1 \times 10^{-6}$ compared with MHCA); 7 of these strains were Amp^s (6 were serotype b), 8 were Amp^{r}NBLP, and 4 were Amp^{r}BLP (2 were serotype b). CHTM medium failed to support the growth of four Amp^{r}NBLP isolates (8%; $P = 0.06$ compared with MHCA). There were no observed differences among the various biotypes for those strains that did not grow.

Disk diffusion tests. When the data from all evaluable strains were pooled, there was no significant difference observed between MHCA, CAE, CHTM, or sBHI medium in the mean zone sizes obtained with the two ampicillin disk tests and the amoxicillin-clavulanate disk test (Table 3). In contrast, PHTM gave statistically significant larger zone sizes compared with those of the other media for all three disk tests ($P < 0.0001$). For example, the mean zone diameter of all organisms tested by the 2-μg ampicillin disk test was 18.2 ± 2.3 (standard error of the mean [SEM]) on PHTM and 13.8 ± 1.7 (SEM) on sBHI. Similarly, the mean zone diameters tested by the 10-μg ampicillin and the amoxicillin-clavulanate disk tests were 27.2 ± 2.5 (SEM) and 38 ± 1.6 (SEM), respectively, on PHTM compared with 18.4 ± 2.3 (SEM) and 28.5 ± 1.0 (SEM) on sBHI. The ability of the disk tests to identify correctly the susceptibilities of the three groups of strains is indicated in Table 4. One Amp^s isolate and two Amp^{r}BLP strains were detected as intermediate by a 10-μg ampicillin disk test on MHCA and PHTM, respectively. None of the Amp^s or Amp^{r}BLP isolates were incorrectly identified as resistant or susceptible, respectively, by any disk test on any of the five media. In contrast, the disk tests with the Amp^{r}NBLP strains with the five media gave variable results. The 10-μg ampicillin disk test misclassified as susceptible or intermediate 6 of 15 strains on sBHI, 6 of 8 strains on PHTM, 5 of 12 strains on CHTM, 6 of 16 strains on MHCA medium, and 7 of 16 on CAE medium. In contrast, the 2-μg ampicillin disk test on sBHI correctly identified all Amp^{r}NBLP strains as resistant, whereas MHCA and CAE media failed to detect as resistant one and two isolates, respectively, and PHTM and CHTM media misclassified as susceptible four and five isolates, respectively. The zone diameter of the two isolates misclas-

TABLE 3. Zone sizes obtained in disk diffusion susceptibility tests[a]

Disk	Medium	Zone diam (mm [range])[b]		
		Amp^s	Amp^{r}BLP	Amp^{r}NBLP
2-μg Ampicillin	sBHI	26 ± 2 (22–29)	6 ± 0 (6)	8 ± 3 (6–14)
	PHTM	33 ± 5 (28–40)	8 ± 3 (6–14)	14 ± 5 (8–24)
	CHTM	25 ± 2 (22–27)	6 ± 1 (6–12)	12 ± 5 (6–21)
	MHCA	25 ± 3 (19–30)	6 ± 0 (6)	10 ± 4 (6–22)
	CAE	25 ± 3 (21–30)	6 ± 0 (6)	9 ± 4 (6–16)
10-μg Ampicillin	sBHI	32 ± 3 (27–36)	6 ± 1 (6–12)	20 ± 4 (13–27)
	PHTM	42 ± 6 (34–55)	16 ± 6 (6–23)	26 ± 6 (20–36)
	CHTM	30 ± 2 (27–34)	8 ± 5 (6–21)	21 ± 4 (14–27)
	MHCA	31 ± 4 (24–36)	7 ± 4 (6–21)	21 ± 5 (12–32)
	CAE	31 ± 4 (25–36)	6 ± 0 (6)	20 ± 4 (12–27)
10-μg Amoxicillin–20-μg clavulanate	sBHI	32 ± 4 (26–38)	29 ± 4 (22–35)	22 ± 4 (16–31)
	PHTM	42 ± 7 (34–54)	39 ± 6 (28–48)	28 ± 7 (22–42)
	CHTM	30 ± 3 (25–33)	30 ± 2 (27–32)	22 ± 3 (17–26)
	MHCA	30 ± 4 (24–34)	30 ± 4 (23–35)	22 ± 4 (15–33)
	CAE	29 ± 4 (24–36)	30 ± 4 (23–35)	22 ± 3 (18–29)

[a] Disk tests were performed as specified by NCCLS publication M2-T4 (9).

[b] A total of 18 Amp^s, 17 Amp^{r}BLP, and 16 Amp^{r}NBLP strains were tested. Not included in the analysis presented here were 1 Amp^{r}NBLP isolate which did not grow on sBHI and 19 of 51 isolates (7 Amp^s, 4 Amp^{r}BLP, and 8 Amp^{r}NBLP) which did not grow on PHTM, and 4 Amp^{r}NBLP strains which did not grow on CHTM. Values are means ± standard deviations.

TABLE 4. Identification of susceptibilities of *H. influenzae* strains by disk tests

Phenotype	No. of isolates[a]																													
	sBHI							PHTM							CHTM						MHCA						CAE					
	Amp^2		Amp^{10}			AMC		Amp^2		Amp^{10}			AMC		Amp^2		Amp^{10}			AMC	Amp^2		Amp^{10}			AMC	Amp^2		Amp^{10}			AMC	
	S	R	S	I	R	S	R	S	R	S	I	R	S	R	S	R	S	I	R	S	R	S	R	S	I	R	S	R	S	R	S	I	R	S	R
Amp^s	18	0	18	0	0	18	0	11	0	11	0	0	11	0	18	0	18	0	0	18	0	18	0	17	1	0	18	0	18	0	18	0	0	18	0
Amp^rBLP	0	17	0	0	17	17	0	0	13	0	2	11	13	0	0	17	0	0	17	17	0	0	17	0	0	17	17	0	0	17	0	0	17	17	0
Amp^rNBLP	0	15	2	4	9	11	4	4	4	5	1	2	8	0	5	7	4	1	7	9	3	1	15	3	3	10	12	4	2	14	3	4	9	12	4

[a] See footnote *b* of Table 3 for explanation of the number of isolates tested. Amp^2, Amp^{10} indicate the 2- and 10-μg ampicillin disk tests, respectively, whereas AMC indicates the 10-μg amoxicillin–20-μg clavulanate disk test. S, Susceptible; R, resistant; I, intermediate. By the 2-μg ampicillin disk test the criterion for resistance was a zone diameter of ≤15 mm and the criterion for susceptibility was a zone diameter of ≥16 mm. By the 10-μg ampicillin disk test, criteria for resistance and susceptibility as specified by NCCLS (9), were as follows: susceptibility, zone diameter of ≥25 mm; intermediate susceptibility, zone diameter of 22 to 24 mm; and resistance, zone diameter of ≤21 mm. By the 10-μg amoxicillin–20 μg clavulanate disk test, the criterion for susceptibility was a zone diameter of ≥20 mm and the criterion for resistance was a zone diameter of ≤19 mm.

sified as susceptible on CAE medium and three of the five isolates misclassified as susceptible on CHTM was 16 mm, which was only 1 mm above our previously recommended cutoff for resistance with the 2-μg ampicillin disk test (4).

DISCUSSION

In contrast to Jorgensen et al. (2), we did not find adequate growth of all *H. influenzae* strains tested on PHTM or CHTM, as 37 and 8% of isolates, respectively, did not grow. In contrast, CAE and MHCA media supported the growth of all 51 strains tested, and sBHI supported the growth of 50 of 51 isolates. The largest phenotypic group of strains that did not grow on PHTM and CHTM were the Amp^rNBLP strains (50 and 25% of isolates, respectively). The lack of growth could be due, in part, to the auxotrophic requirements of these strains (5); however, regional differences among Amp^s and Amp^rBLP strains appear to be unlikely as an explanation for the paradox between the results of this investigation and those of Jorgensen et al. (2). Different lots of Mueller-Hinton base might be implicated; of note, we and Jorgensen et al. (2) used the same vendor (Difco). Of interest, commercially prepared HTM plates from Prepared Media Laboratories were not available, as production was delayed while different lots of Mueller-Hinton base and other ingredients were tested in an attempt to obtain reproducible standardization (Gary Wicklund, personal communication). In addition, Remel Laboratories, which has released HTM plates for clinical use, formulated CHTM with Mueller-Hinton base obtained from a different vendor. Because the CHTM plates supported the growth of the 19 strains that did not grow on PHTM plates in this study, one might conclude that there may be some nutritional difference between these two bases. However, confirmation would require direct comparison with all other ingredients being identical. Nevertheless, the inability of HTM to support the growth of *H. influenzae* coupled with a more than threefold increase in the cost per CHTM plate compared with the cost of MHCA or CAE medium makes the adoption of this new method unjustified.

Suggestive of the reduced growth on HTM, Jorgensen et al. (2) observed at least 1 $\log_2$ dilution lower MICs for cephalothin, cefaclor, rifampin, and tetracycline when they compared HTM and Mueller-Hinton chocolate medium (2). In addition, their geometric mean MICs were lower for all 9 antimicrobial agents tested by agar dilution and for 9 of 10 antimicrobial agents (excluding trimethoprim-sulfamethoxazole) tested by the broth dilution technique, which led to their proposed interpretative zones, which were all larger than those previously recommended by NCCLS for MHCA medium (2, 7). Although our MIC data were not determined at an inoculum of 10^4 CFU as recommended by NCCLS (8), even at the higher inoculum of 10^5 CFU, resistant strains appeared to be susceptible on PHTM.

Use of PHTM and CHTM media and the standard 10-μg disk with the new NCCLS criteria for disk tests identified all Amp^s strains as susceptible but misclassified 8 and 5 evaluable resistant strains, respectively, as intermediate or susceptible (Table 4). Although both ampicillin disk tests on CHTM identified all Amp^rBLP strains as resistant, on PHTM, 2 of 13 strains (15%) appeared to be of intermediate susceptibility (zone diameter, 22 to 24 mm). Misclassification on either PHTM or CHTM was most striking with the Amp^rNBLP strains; 4 of 12 strains (33%) and 5 of 8 strains (62%) appeared to be susceptible on CHTM and PHTM, respectively, while one additional isolate on each appeared to be of intermediate susceptibility. Thus, 42% (5 of 12) and 75% (6 of 8) of Amp^rNBLP strains were not identified as resistant with CHTM or PHTM medium, respectively. These data are consistent with our previous inability to detect 44% (8 of 18) of these isolates by using the former NCCLS criteria and media (MHCA) and the 10-μg ampicillin disk test. However, the lack of growth on the previously approved media was not observed. Official adoption of HTM as the specific medium for *H. influenzae* susceptibility testing and of the new NCCLS guidelines appears to be problematic, given these disturbing data which show the inability of HTM to support the growth of all *H. influenzae* and the misclassification of resistant strains as susceptible.

Unless there is a routine disk test that will detect Amp^rNBLP strains reliably, their incidence will remain underestimated. Given their intermediate level of ampicillin resistance compared with those of β-lactamase-producing strains, it seems apparent that use of the lower-concentration, 2-μg ampicillin disk test would best detect this group and also allow for easy identification of Amp^rBLP strains. Our data show that Amp^rBLP strains would grow close to the disk (mean zone diameter, 6 mm), Amp^s isolates would have large zones (mean zone diameter, 25 mm), and Amp^rNBLP strains would have zones of approximately 10 mm. PHTM and CHTM media would be unacceptable even with the 2-μg disk test, as 4 of 8 (50%) and 5 of 12 (42%) Amp^rNBLP strains, respectively, were misclassified as susceptible. If the cutoff were increased to ≤16 mm for resistance from our previously recommended criteria of ≤15 mm, the 2-μg ampicillin disk test would have correctly identified Amp^rNBLP strains tested on sBHI (15 of 15), CAE medium (16 of 16), or MHCA (15 of 16) medium; on CHTM and

PHTM, 2 of 12 and 4 of 8 strains, respectively, would have been misclassified as resistant. Further investigation with the 2-μg ampicillin disk test in other laboratories with media that support the growth of *H. influenzae* strains would clarify the exact zone size cutoff which would correctly identify resistant and susceptible strains.

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