JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1992, p. 2284–2289
0095-1137/92/092284-06$02.00/0

Vol. 30, No. 9

Broth Microdilution Testing of *Haemophilus influenzae* with Haemophilus Test Medium versus Lysed Horse Blood Broth

S. R. SCRIVER,[1] D. E. LOW,[1,2,3,4]* A. E. SIMOR,[1,2,3] B. TOYE,[1,3]† A. McGEER,[1,2,3] R. JAEGER,[4] AND CANADIAN HAEMOPHILUS STUDY GROUP‡

Departments of Microbiology, Mount Sinai Hospital[1] and Princess Margaret Hospital,[2] University of Toronto,[3] and MDS Laboratories,[4] Toronto, Ontario M5G 1X5, Canada

Received 20 March 1992/Accepted 16 June 1992

Broth microdilution testing of 702 community-acquired isolates of *Haemophilus influenzae* from across Canada was performed with both Mueller-Hinton broth supplemented with 3% lysed horse blood broth (LHB) (BBL Microbiology Systems, Cockeysville, Md.) and haemophilus test medium (HTM). The prevalence of β-lactamase production was found to be 26% with no regional variation. MICs determined with LHB tended to be higher than those with HTM, but interpretive errors due to these differences were observed only rarely with trimethoprim-sulfamethoxazole (n = 5), cefaclor (n = 8), and cefamandole (n = 3). The interobserver variability in MIC determinations was found to be greater when LHB was used than when HTM was used. There was no difference in intraobserver variability between the two medium formulations. β-Lactamase-positive isolates developed false resistance to amoxicillin-clavulanate 2 weeks after microdilution panels of both types of medium were stored at −20°C but not when panels were stored at −70°C. In conclusion, this study supports the use of HTM rather than LHB for sensitivity testing of *H. influenzae* because of its lower rate of interobserver variability and its ability to support the growth of these organisms, which is comparable to that of LHB.

Haemophilus influenzae is an important pathogen in upper and lower respiratory tract infections, meningitis, and septicemia (21, 35). Increased antimicrobial resistance has necessitated the use of growth-based susceptibility testing (5, 7, 13, 32). Since conventional media do not support the growth of *Haemophilus* spp., accepted standard susceptibility test media cannot be used. As a result, more than 30 different agar and broth formulations have been evaluated (3). Prior to 1990, the National Committee for Clinical Laboratory Standards (NCCLS) recommended the use of cation-supplemented Mueller-Hinton broth with 3% lysed horse blood (LHB) (27). LHB, although it provides the necessary growth requirements, is opaque and therefore difficult to read, antagonistic to certain antimicrobial agents, and not available commercially. Because of these inherent problems, the M7-A2 NCCLS guidelines (28) have recommended the use of haemophilus test medium (HTM) (17). HTM is optically clear and nonantagonistic, and it can be used in both broth and agar formulations (17). However, there have been very few studies evaluating the use of HTM (9, 16, 24, 25). We therefore conducted a study to compare in vitro susceptibility testing of *H. influenzae* in HTM with that in LHB. The reproducibility with which MIC determinations were made with both media was also determined. In addition, because a previous study had suggested that amoxicillin-clavulanate may be unstable in HTM (34), we evaluated the stability of this antimicrobial agent in both HTM and LHB.

MATERIALS AND METHODS

In vitro susceptibility testing. A total of 702 *H. influenzae* strains isolated from community-acquired infections from eight centers across Canada were used in this study. Isolates were identified as *H. influenzae* by standard methodology (18). Isolates were frozen at −70°C in buffered glycerol and subcultured twice prior to susceptibility testing. Subcultures were performed with chocolate agar plates (Difco, Detroit, Mich.) and incubated at 37°C in 5% CO_2 for 16 to 18 h.

β-Lactamase production was detected with a nitrocefin-impregnated disk (Cefinase; BBL Microbiology Systems, Cockeysville, Md.). HTM was prepared as described by Jorgensen et al. (17) with 15 μg of hematin per ml (Sigma Chemical Co., St. Louis, Mo.), 5 mg of yeast extract per ml (Difco), and 15 μg of NAD per ml (Sigma) added to cation-supplemented Mueller-Hinton broth (BBL). LHB was prepared by using cation-supplemented Mueller-Hinton broth (BBL) with 3% lysed horse blood (Woodlyn Laboratories, Guelph, Ontario, Canada) and 10 μg of β-NAD per ml (27).

Microdilution broth testing was performed according to NCCLS guidelines (28). Antibiotic panels were prepared by dispensing media containing twofold-concentration increments of antimicrobial agents in 100-μl volumes into plastic, 96-well trays (Dynatech Laboratories, Alexandria, Va.). The same antimicrobial stock solutions were used to make both types of panels. Thymidine phosphorylase (Sigma) was aseptically added to wells containing trimethoprim-sulfamethoxazole to achieve a final concentration of 0.2 IU/ml

* Corresponding author.
† Present address: Department of Microbiology, Ottawa General Hospital, Ottawa, Ontario, Canada.
‡ M. Dalton, Victoria General Hospital, Halifax, Nova Scotia, Canada; P. Doyle, Metro-McNair Clinical Laboratories, Vancouver, British Columbia, Canada; S. M. Gubash, Provincial Laboratory of Public Health, Calgary, Alberta, Canada; G. Horsman, H. E. Robertson Laboratory, Saskatchewan Health, Laboratory and Disease Control Services Branch, Regina, Saskatchewan, Canada; M. Lovgren, Provincial Laboratory of Public Health for Northern Alberta, Edmonton, Alberta, Canada; R. Rennie,§ Royal University Hospital, Saskatoon, Saskatchewan, Canada; and T. Williams, Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada.
§ Present address: Department of Microbiology, University of Alberta Hospital, Edmonton, Alberta, Canada.

(28). Growth from a 16- to 18-h culture was suspended in 3 ml of Mueller-Hinton broth and diluted to match the turbidity of a 0.5 McFarland standard with a turbidimeter (A-Just; Abbott Diagnostics, Abbott Park, Ill.). Inocula were further diluted and added to the microdilution trays to achieve a final inoculum of 5×10^5 CFU/ml. Panels of both types were inoculated with the same 0.5 McFarland standard for each organism. After the panel was inoculated, a 10-μl sample was removed from the growth control well and diluted 1:500 with Mueller-Hinton broth. An aliquot of 100 μl of the diluted suspension was inoculated onto a chocolate agar plate and, after an overnight incubation, a colony count was performed (2). *H. influenzae* MICs were determined after incubation at 35°C for 24 h for LHB and 20 to 24 h for HTM. *H. influenzae* ATCC 49247 was used as a control organism for susceptibility testing.

A very major error occurred when a strain was found to be resistant by HTM but susceptible by LHB. A major error occurred when a strain was found to be susceptible by HTM but determined to be resistant by LHB. All other discrepancies in interpretation were called minor errors.

Reproducibility of MIC determination. All technologists reviewed the guidelines set by the NCCLS for the reading and interpretation of MICs in broth microdilution panels prior to testing (28). All panels were coded so that the technologists were blind to strain identities. Interobserver variability was determined by having five technologists each read 20 different panels of LHB and 5 different panels of HTM. Each MIC determination for each antimicrobial agent in each panel was compared with MIC determinations carried out in a single-blinded fashion by the four other technologists. Intraobserver variability was determined by having three technologists read five different microdilution panels of each medium in duplicate. Each MIC determination for each panel was compared with the second reading by the same technologist, and the difference between the two readings was determined.

Each paired comparison was assessed and graded as to the difference (in number of wells) between the two determinations. Differences were categorized as insignificant or significant. Insignificant errors were those in which the difference between two MIC determinations was <3 dilutions; significant errors were those in which the difference between the two MIC determinations was ≥3 dilutions. A significant error was defined as a ≥3-dilution difference because a difference of ±1 dilution, which encompasses a 2-dilution range, is considered an acceptable difference (28). Paired comparisons in which the difference could not be categorized because one or both of the MICs were out of the range of the dilutions tested were excluded. The proportions of significant errors for each antibiotic were then compared by using the Fisher exact test or the Yates corrected chi-square test (11). An overall comparison of the two media was carried out by using a Mantel-Haenszel analysis considering each antimicrobial agent as one stratum (22).

Clavulanic acid stability. The stability of clavulanic acid was assessed by determining the in vitro susceptibilities of 10 isolates of *H. influenzae* to amoxicillin-clavulanic acid at weekly intervals for 8 weeks. Of the 10 isolates, 5 were β-lactamase producers, including *H. influenzae* ATCC 35056, 4 were non-β-lactamase producers, including *H. influenzae* ATCC 10211, and one was ampicillin-resistant, β-lactamase-negative *H. influenzae* ATCC 49247. All isolates were tested with both panels stored at either −20°C or −70°C at weekly intervals for the test period.

RESULTS

In vitro susceptibility testing. The 702 strains of *H. influenzae* were community-acquired isolates from eye (*n*, 357), sputum (*n*, 205), sterile sites (*n*, 58), and other sites (*n*, 82). A total of 182 (26%) of the 702 strains were determined to be β-lactamase positive. The prevalence of β-lactamase production did not vary significantly from region to region.

All 702 isolates of *H. influenzae* were tested with both HTM and LHB. Initially, 27 (3.9%) of the 702 isolates failed to grow in HTM; however, 24 of these grew upon repeated testing. Five (0.7%) of the 702 isolates failed to grow initially in LHB; however, 4 out of these 5 isolates grew upon repeated testing. There were no isolates that failed to grow in both medium formulations. The four isolates that failed to grow in either HTM (3) or LHB (1) did not demonstrate CO_2 dependency and were excluded from further study, so that 698 isolates were included in the medium comparison. When the inoculum density was estimated by performing colony counts, the inoculum was found to vary between 2×10^5 and 18×10^5 CFU/ml.

Of the 516 β-lactamase-negative isolates, 3 (0.6%) were found to be resistant to ampicillin, with MICs of 2.0 μg/ml in HTM and 4.0 μg/ml in LHB (Table 1). None of the β-lactamase-negative isolates, including the β-lactamase-negative, ampicillin-resistant isolates, were resistant to the cephalosporins tested. The variation between the MICs determined with LHB and those determined with HTM for these isolates is presented in Table 2. Minor errors were found with only four antimicrobial agents: ampicillin (*n*, 2), cefaclor (*n*, 13), trimethoprim-sulfamethoxazole (*n*, 11), and erythromycin (*n*, 40). Very major errors existed only for trimethoprim-sulfamethoxazole (*n*, 5).

The MICs determined for the 182 β-lactamase-producing *H. influenzae* strains are also shown in Table 1. For β-lactamase-positive strains, there were 3 major errors (all with cefaclor), and 11 minor errors (8 with cefaclor and 3 with cefamandole). The variation between MICs determined with LHB and those determined with HTM is presented in Table 2.

Clavulanic acid stability. All of the β-lactamase-negative, ampicillin-sensitive isolates remained sensitive to amoxicillin-clavulanic acid when both types of medium were stored at either −20°C or −70°C. *H. influenzae* ATCC 49247 did have a MIC to amoxicillin-clavulanate in the resistant range (>8 μg/ml) during the test period, but all MICs remained within the acceptable quality control range (2 to 16 μg/ml). Three β-lactamase-positive isolates showed resistance to amoxicillin-clavulanic acid at 2 weeks, and five showed resistance by week 4 when both panels containing HTM and those containing LHB were stored at −20°C. All isolates tested from panels that had been stored at −70°C remained susceptible.

Reproducibility of MIC determinations. Table 3 shows the results of interobserver variability by presenting the occurrence of significant errors as a percentage of the total number of paired comparisons. There was a higher number of significant errors with LHB for four of the five antimicrobial agents evaluated. The Mantel-Haenszel weighted relative risk for significant errors with LHB compared with HTM was 2.11 (95% confidence limits, 1.37 to 3.25).

In determining intraobserver variability, we found that the number of significant errors that occurred was not different for the two media. One reader made two significant errors with panels containing HTM. A different reader made five significant errors with panels containing LHB.

TABLE 1. In vitro activity of several antimicrobial agents against 516 β-lactamase-negative and 182 β-lactamase-positive *H. influenzae* isolates

Antimicrobial agent and β-lactamase test result[a]	MIC (μg/ml)[b]					
	LHB			HTM		
	Range	50%	90%	Range	50%	90%
Ampicillin						
Neg	0.125–4.0	0.5	1.0	0.125–2.0	0.5	1.0
Pos	2.0–>128	32	128	2.0–>128	16	128
Amoxicillin-clavulanate						
Neg	≤0.5–2.0	≤0.5	1.0	≤0.50–1.0	≤0.5	0.5
Pos	≤0.5–2.0	≤0.5	1.0	≤0.50–1.0	≤0.5	1.0
Cefaclor						
Neg	≤0.25–16.0	2.0	4.0	≤0.25–16.0	2.0	4.0
Pos	≤0.25–64.0	4.0	8.0	≤0.25–32.0	2.0	4.0
Cefamandole						
Neg	≤0.25–8.0	≤0.25	1.0	≤0.25–4.0	0.5	1.0
Pos	≤0.25–16.0	0.5	2.0	≤0.25–16.0	0.5	2.0
Cefuroxime						
Neg	≤0.25–2.0	0.5	0.5	≤0.25–4.0	≤0.25	0.5
Pos	≤0.25–8.0	0.5	1.0	≤0.25–4.0	0.5	1.0
Erythromycin						
Neg	≤0.5–8.0	2.0	4.0	≤0.5–8.0	2.0	4.0
Pos	≤0.5–8.0	2.0	4.0	≤0.5–8.0	0.5	4.0
Chloramphenicol						
Neg	≤2.0	≤2.0	≤2.0	≤2.0	≤2.0	≤2.0
Pos	≤2.0	≤2.0	≤2.0	≤2.0	≤2.0	≤2.0
Trimethoprim-sulfamethoxazole						
Neg	≤0.5–2.0	≤0.5	≤0.5	≤0.5–4.0	≤0.5	≤0.5
Pos	≤0.5–2.0	≤0.5	≤0.5	≤0.5–4.0	≤0.5	≤0.5

[a] Neg, negative; pos, positive.
[b] 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

DISCUSSION

Prior to 1970, *H. influenzae* was universally susceptible to ampicillin, tetracycline, and chloramphenicol (30). In 1972, ampicillin resistance due to β-lactamase production was first reported (12). Since then, surveys of *H. influenzae* have also reported resistance to chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, erythromycin, cefaclor, and rifampin (4, 5, 7, 20, 36). Ampicillin-resistant, β-lactamase-negative isolates have also been documented (23). Although rapid tests for β-lactamase and chloramphenicol acetyltransferase detect resistance to ampicillin due to β-lactamase production and to chloramphenicol (1, 12), growth-based susceptibility testing is necessary to detect ampicillin-resistant, β-lactamase-negative isolates and isolates resistant to other antimicrobial agents (15). The 26% prevalence of β-lactamase-producing *H. influenzae* isolates found in this study is similar to that reported in other studies in Canada (17%) (4) and in the United States (20%) (8) but higher than that reported in Europe (10%) (20). A survey of *H. influenzae* isolates from nonhospitalized patients in Ontario, Canada; New York; and Pennsylvania found a prevalence of β-lactamase production of 24% (14). The prevalence of ampicillin-resistant, β-lactamase-negative strains determined in this study (<0.1%) was much lower than that determined by Bergeron (3) in 1987 (2.6%) but similar to that found by Doern et al. (7) in 1986 (0.1%).

The methodology for the in vitro susceptibility testing of *H. influenzae* remains controversial (30). Standard conditions which have been developed for other microorganisms cannot be used because of the fastidious growth requirements of *Haemophilus* spp. In vitro susceptibility studies using different media, inocula, and incubation atmospheres have all demonstrated variable activities for the same antimicrobial agent, since all of these factors may influence results. The previously recommended formulation for susceptibility testing of *H. influenzae* was LHB. This medium is opaque and antagonistic to certain drugs, and it lacks consistency and may not be available commercially. It was hoped that the adoption of HTM by the NCCLS would resolve these difficulties.

In this study, the potential variation between HTM and LHB was minimized by deriving the panels from the same antimicrobial stock solutions and inoculating both panels with the same suspension of organisms. Although most organisms grew well in both media, some isolates failed to grow initially. Much anecdotal evidence that HTM may not reliably and reproducibly support the growth of all strains of *H. influenzae* exists (6). The occurrence of poor growth or actual growth failures with HTM may be due to different lots of Mueller-Hinton base (26) or an erratic V-factor inhibitor (3). More likely, inadequate growth may be due to the X-factor content of the medium, since hematin, or X factor,

TABLE 2. Comparison of MICs determined with LHB with those determined with HTM when 516 β-lactamase-negative and 182 β-lactamase-positive *H. influenzae* isolates were tested

Antimicrobial agent and β-lactamase test result[a]	No. of LHB MICs within the following $\log_2$ dilution of HTM MICs (%)						
	≥−3	−2	−1	0[b]	1	2	≥3
Ampicillin							
Pos	3 (2)	3 (2)	20 (11)	60 (33)	46 (25)	30 (16)	20 (11)
Neg	0 (0)	0 (0)	67 (13)	288 (56)	130 (25)	31 (16)	0 (0)
Amoxicillin-clavulanate							
Pos	0 (0)	2 (1)	46 (25)	102 (56)	30 (16)	1 (1)	1 (1)
Neg	0 (0)	2 (1)	51 (10)	273 (53)	185 (36)	5 (1)	0 (0)
Cefaclor							
Pos	1 (1)	1 (11)	13 (7)	72 (40)	56 (31)	26 (14)	13 (7)
Neg	0 (0)	60 (12)	168 (33)	211 (41)	70 (14)	7 (1)	0 (0)
Cefamandole							
Pos	6 (3)	11 (6)	45 (25)	64 (35)	37 (20)	12 (7)	7 (4)
Neg	0 (0)	0 (0)	31 (6)	325 (63)	155 (30)	5 (1)	0 (0)
Cefuroxime							
Pos	1 (1)	2 (1)	38 (21)	104 (57)	29 (16)	6 (3)	2 (1)
Neg	0 (0)	4 (1)	65 (13)	294 (57)	148 (29)	5 (1)	0 (0)
Erythromycin							
Pos	0 (0)	3 (2)	22 (12)	89 (49)	51 (28)	17 (9)	0 (0)
Neg	0 (0)	0 (0)	67 (13)	243 (47)	175 (34)	31 (6)	0 (0)
Chloramphenicol							
Pos	0 (0)	0 (0)	0 (0)	182 (100)	0 (0)	0 (0)	0 (0)
Neg	0 (0)	0 (0)	0 (0)	516 (100)	0 (0)	0 (0)	0 (0)
Trimethoprim-sulfamethoxazole							
Pos	0 (0)	3 (2)	3 (2)	175 (96)	0 (0)	1 (1)	0 (0)
Neg	0 (0)	7 (1)	0 (0)	506 (98)	0 (0)	3 (1)	0 (0)

[a] Pos, positive; neg, negative.
[b] MICs were the same in LHB and HTM.

is poorly soluble and extremely unstable, and there are isolates of *H. influenzae* with high growth requirements for X factor (6). However, none of the above reasons can fully explain our findings, since most isolates that didn't grow initially did grow when they were retested on the same lot of microtiter plates. New NCCLS guidelines (M100-S3) (29) have recommended the use of two additional American Type Culture Collection strains of *H. influenzae* for the testing of HTM. One of these, *H. influenzae* ATCC 10211, which has a high growth requirement for hematin, has been recommended as a control strain to verify the growth-promotional properties of HTM.

TABLE 3. Results of interobserver variability of MIC determinations with LHB and HTM

Antimicrobial agent	% Significant difference[a] for the following media		*P* value
	LHB[b]	HTM[c]	
Ampicillin	29	12	0.03
Amoxicillin-clavulanate	11	22	0.07
Cefaclor	11	0	0.01
Cefamandole	8	0	0.05
Cefuroxime	15	0	0.01

[a] Significant difference, interobserver variability of ≥3 dilutions.
[b] 200 paired comparisons.
[c] 50 paired comparisons.

MICs determined with LHB were found to be slightly higher than the respective MICs determined with HTM. This may be explained by the fact that LHB is nutritionally very rich, whereas HTM supplies only the basic requirements necessary for the growth of *Haemophilus* spp. The major errors found when trimethoprim-sulfamethoxazole was tested are not unexpected, because of the inherent difficulties in performing trimethoprim-sulfamethoxazole MIC determinations with blood containing media (31).

There are multiple problems that exist in testing and interpreting results for erythromycin (2). Barry et al. (2) found that, by the current NCCLS M7-A2 guidelines, strains found to be resistant in LHB were mostly moderately susceptible in HTM. This lack of correlation between HTM and LHB when macrolides were tested was also found in this study. Until this issue is resolved, it has been suggested that erythromycin should not be tested against *H. influenzae* in the routine laboratory (25).

The major errors encountered with cefaclor and cefamandole may be due to inoculum variation, since both of these antimicrobial agents are known to be sensitive to small variances in inoculum (30). Ideally, the inoculum should vary only within one-half log concentration of the target concentration. Therefore, if the desired final concentration is 5×10^5 CFU/ml, the inoculum range should fall between $1 \times$

10^5 and 10×10^5 CFU/ml. However, many authors have found when testing *H. influenzae* that there is a wide range of colony counts when a turbidity standard equivalent to a 0.5 McFarland is used. Lapoint and Lavellee (19) found that, despite their use of a turbidometer, 72% of the colony counts were $>10^8$ CFU/ml. Barry et al. (2) and Fernandez et al. (10) also found this in their studies.

In determining interobserver variability, it was found that significant errors in MIC determinations occurred more frequently with LHB than with HTM. The transparency of HTM simplifies endpoint determination. It is, however, important to note that significant errors did occur with both types of medium, with all antibiotics, and with all five technologists.

NCCLS M7-A2 guidelines state that when broth microdilution panels are stored, the user should store them in a frozen state (preferably at −60 or −70°C) and thaw them as needed (28). In this study, it was found that any period of storage longer than 2 weeks at −20°C of HTM or LHB panels containing amoxicillin-clavulanate would result in the appearance of false resistance in β-lactamase-producing strains of *H. influenzae*. This artifact would not be detected by the control strain, *H. influenzae* ATCC 49247, since it is an ampicillin-resistant, β-lactamase-negative strain and is therefore unaffected by the β-lactamase inhibitor clavulanic acid. We propose that the NCCLS guidelines be amended to contain the provision that broth microdilution panels containing amoxicillin-clavulanate should be stored only at −70°C. However, if this presents a problem to those laboratories without the appropriate freezers, a β-lactamase-positive control organism should be included to detect such false resistance.

ACKNOWLEDGMENTS

We thank Elizabeth Konigshaus and Alice Au Yeung for their excellent secretarial services and Keith O'Rourke for his assistance in the statistical analysis. We also thank Steven Lai, Pauline Lo, T. Christine Moore, Shirley Szeto, and Kelly Tsimidis for their technical assistance.

REFERENCES

1. **Azemun, P., T. Stull, M. Roberts, and A. L. Smith.** 1981. Rapid detection of chloramphenicol resistance in *Haemophilus influenzae*. Antimicrob. Agents Chemother. **20**:168–170.
2. **Barry, A. L., P. B. Fernandes, J. H. Jorgensen, C. Thornsberry, D. J. Hardy, and R. N. Jones.** 1988. Variability of clarithromycin and erythromycin susceptibility tests with *Haemophilus influenzae* in four different broth media and correlation with the standard disk diffusion test. J. Clin. Microbiol. **26**:2415–2420.
3. **Bergeron, M. G., P. Simard, and P. Provencher.** 1987. Influence of growth medium and supplement on growth of *Haemophilus influenzae* and on antibacterial activity of several antibiotics. J. Clin. Microbiol. **25**:650–655.
4. **Bergeron, M. G., L. D. Tremblay, J. L'Ecuyer, P. Provencher, and The Canadian Study Group.** 1990. Susceptibility of *Haemophilus influenzae* to antimicrobial agents used in Canada. Can. Med. Assoc. J. **143**:895–901.
5. **Campos, J., S. Garcia-Tornel, and I. Sanfeliu.** 1984. Susceptibility studies of multiply resistant *Haemophilus influenzae* isolated from pediatric patients and contacts. Antimicrob. Agents Chemother. **25**:706–709.
6. **Doern, G. V.** Newly adopted NCCLS recommendations for in vitro susceptibility testing of *Haemophilus influenzae*. Submitted for publication.
7. **Doern, G. V., J. H. Jorgensen, C. Thornsberry, D. A. Preston, and the *Haemophilus influenzae* Surveillance Group.** 1986. Prevalence of antimicrobial resistance among clinical isolates of *Haemophilus influenzae*: a collaborative study. Diagn. Microbiol. Infect. Dis. **4**:95–107.
8. **Doern, G. V., J. H. Jorgensen, C. Thornsberry, D. A. Preston, T. Tubert, J. S. Redding, and L. A. Maher.** 1988. National collaborative study of the prevalence of antimicrobial resistance among clinical isolates of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **32**:180–185.
9. **Doern, G. V., J. H. Jorgensen, C. Thornsberry, and H. Snapper.** 1990. Disk diffusion susceptibility testing of *Haemophilus influenzae* using Haemophilus Test Medium. Eur. J. Clin. Microbiol. Infect. Dis. **9**:329–336.
10. **Fernandez, P. B., D. Hardy, R. Bailer, E. McDonald, J. Pinter, N. Ramer, R. Swanson, and E. Gade.** 1987. Susceptibility testing of macrolide antibiotics against *Haemophilus influenzae* and correlation of in vitro results with in vivo efficacy in a mouse septicemia model. Antimicrob. Agents Chemother. **31**:1243–1250.
11. **Fleiss, J. L.** 1981. Statistical methods for rates and proportions, 2nd ed., p. 19–32. John Wiley and Sons, New York.
12. **Gunn, B. A., J. B. Woodall, J. F. Jones, and C. Thornsberry.** 1974. Ampicillin-resistant *Haemophilus influenzae*. Lancet **ii**: 845.
13. **Hansen, D.** 1975. *Haemophilus influenzae* type b resistant to tetracycline. Lancet **i**:893–896.
14. **Jaeger, R., and D. E. Low.** 1987. Prevalence of ampicillin-resistant *Haemophilus influenzae* isolated from non-hospitalized patients in Ontario, New York and Pennsylvania. Can. Dis. Weekly Rep. **13**:183.
15. **Jorgensen, J. H.** 1989. Antimicrobial susceptibility testing of *Haemophilus influenzae*: an update. Antimicrob. Newsl. **6**:65–68.
16. **Jorgensen, J. H., A. W. Howell, and L. A. Maher.** 1990. Antimicrobial susceptibility testing of less commonly isolated *Haemophilus* species using *Haemophilus* test medium. J. Clin. Microbiol. **28**:985–988.
17. **Jorgensen, J. H., J. S. Redding, L. A. Maher, and A. W. Howell.** 1987. Improved medium for antimicrobial susceptibility testing of *Haemophilus influenzae*. J. Clin. Microbiol. **25**:2105–2113.
18. **Kilan, M.** 1991. *Haemophilus*, p. 463–470. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
19. **Lapoint, J. R., and C. Lavellee.** 1987. Antibiotic interaction of amoxycillin and clavulanic acid against 132 β-lactamase positive *Haemophilus influenzae* isolates; a comparison with some other oral agents. J. Antimicrob. Chemother. **19**:49–58.
20. **Machka, K., I. Braveny, H. Dabernat, K. Dornbusch, E. Van Dyck, F. H. Kayser, B. Van Klingeren, H. Mittermayer, E. Perea, and M. Powell.** 1988. Distribution and resistance patterns of *Haemophilus influenzae*: a European cooperative study. Eur. J. Clin. Microbiol. Infect. Dis. **7**:14–24.
21. **Mäkelä, P. H.** 1988. Unencapsulated *Haemophilus influenzae*—what kind of pathogen? Eur. J. Clin. Microbiol. Infect. Dis. **7**:606–609. (Editorial.)
22. **Mantel, N., and W. Hentzel.** 1959. Statistical aspects of the analysis of data from retrospective studies of disease. J. Natl. Cancer Inst. **22**:719–748.
23. **Markowitz, S. M.** 1980. Isolation of an ampicillin-resistant, non-β-lactamase-producing strain of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **17**:80–83.
24. **Marsik, F., G. Evans, L. Thompson, and J. Fowler.** 1991. Comparison of Haemophilus Test Medium and Mueller-Hinton broth containing lysed horse blood and NAD for microdilution susceptibility testing of *Haemophilus influenzae*, abstr. C-127, p. 363. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.
25. **Mendelman, P. M., D. O. Chaffin, C. Clausen, T. L. Stull, C. I. Needham, J. D. Williams, and A. L. Smith.** 1986. Failure to detect ampicillin-resistant, non-β-lactamase-producing *Haemophilus influenzae* by standard disk susceptibility testing. Antimicrob. Agents Chemother. **30**:274–280.
26. **Mendelman, P. M., E. A. Wiley, T. L. Stull, C. Clausen, D. O. Chaffin, and O. Önay.** 1990. Problems with current recommendations for susceptibility testing of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **34**:1480–1484.

27. **National Committee for Clinical Laboratory Standards.** 1985. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A. National Committee for Clinical Laboratory Standards, Villanova, Pa.
28. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
29. **National Committee for Clinical Laboratory Standards.** 1991. Performance standards for antimicrobial susceptibility testing; 3rd information supplement. NCCLS document M100-S3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
30. **Needham, C. A.** 1988. *Haemophilus influenzae*: antibiotic susceptibility. Clin. Microbiol. Rev. **1**:218–227.
31. **Sindi, R., S. Hammerberg, M. I. Marks, and C. H. Pai.** 1978. In vitro susceptibility of *Haemophilus influenzae* to sulfamethoxazole-trimethoprim and cefaclor, cephalexin, and cephadrin. Antimicrob. Agents Chemother. **13**:861–864.
32. **Strandberg, D. A., J. H. Jorgensen, and D. J. Drutz.** 1984. Activities of newer β-lactam antibiotics against ampicillin, chloramphenicol, or multiply resistant *Haemophilus influenzae*. Diagn. Microbiol. Infect. Dis. **2**:333–337.
33. **Syriopoulou, V. P., D. W. Scheifele, C. M. Sack, and A. L. Smith.** 1979. Effect of inoculum size on the susceptibility of *Haemophilus influenzae* b to β-lactam antibiotics. Antimicrob. Agents Chemother. **16**:510–513.
34. **Toye, B., S. Fuller, A. E. Simor, A. McGeer, and D. E. Low.** 1990. Susceptibility testing of β-lactamase-positive *Haemophilus influenzae* using Haemophilus Test Medium, abstr. C-270, p. 389. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990. American Society for Microbiology, Washington, D.C.
35. **Turk, D. C.** 1984. The pathogenicity of *Haemophilus influenzae*. J. Med. Microbiol. **18**:1–16.
36. **Van Klingeren, B., J. D. A. Embden, and M. Dessens-Kroon.** 1977. Plasmid-mediated chloramphenicol resistance in *Haemophilus influenzae*. Antimicrob. Agents Chemother. **11**:383–387.
37. **Williams, J. D., and F. Moosdeen.** 1986. Antibiotic resistance in *Haemophilus influenzae*: epidemiology, mechanisms, and therapeutic possibilities. Rev. Infect. Dis. **8**(Suppl. 5):S555–S561.