

Influences of Media and Inocula on the In Vitro Susceptibility of *Haemophilus influenzae* to Co-Trimoxazole, Ampicillin, Penicillin, and Chloramphenicol

MELVIN I. MARKS* AND GERRY WEINMASTER

Department of Pediatrics (Infectious Diseases), McGill University, and McGill University-Montreal Children's Hospital Research Institute,* Montreal 108, Quebec, Canada

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The effects of inocula and media on the activities of ampicillin, penicillin, chloramphenicol and co-trimoxazole against *Haemophilus influenzae* were examined in vitro. Two inocula and four media were tested by the disk diffusion, broth dilution, and agar dilution methods. Chloramphenicol activity versus *H. influenzae* was least affected by changes in inocula and media, whereas co-trimoxazole was most susceptible to these effects. Filde's and Levinthal's agar dilution tests were most satisfactory for ampicillin. Penicillin was less active on Levinthal's than on Filde's agar. Both ampicillin and penicillin were less active when tested against the higher inoculum. Co-trimoxazole was most active (<1% *H. influenzae* was resistant) when tested at an inoculum of 10^8 colony-forming units/ml on diagnostic susceptibility test agar with 5% lysed horse blood added. The majority of *H. influenzae* appeared resistant to co-trimoxazole with increases in the test inocula and/or when tested on brain heart infusion with Filde's, Levinthal's or "low-thymidine" Mueller-Hinton medium.

The recognition of *Haemophilus influenzae* resistant to ampicillin has emphasized the importance of routine susceptibility testing of these bacteria in hospital laboratories (6, 11, 15). A study was undertaken to evaluate the comparative antimicrobial susceptibilities of *H. influenzae* to four currently available antimicrobials and to determine the effects of inocula and media on these in vitro measurements.

MATERIAL AND METHODS

H. influenzae were identified by Gram stain, colonial morphology, and X- and V-factor growth requirements. Nitrate reduction and indole production tests were done on nontypable eye isolates (8). Typing was performed by the slide agglutination technique using commercial type-specific antisera (Difco) to *H. influenzae*.

Strains were isolated from ill children seen at the Montreal Children's Hospital from 1 January to 31 October 1974 and were stored in pure culture in defibrinated horse blood at -70°C . The sources and respective number of *H. influenzae* studied were as follows: blood, 17; cerebrospinal fluid, 13; eye, 20; nose, 2; sputum, 3; throat, 7; pleural fluid, 2; ear, 1; nasopharynx, 5; wound, 2. Seventy-two isolates were tested for susceptibility to ampicillin, penicillin, chloramphenicol, and co-trimoxazole (trimethoprim [TMP]/sulfamethoxazole [SMZ]) by the disk diffusion, broth dilution, and agar dilution methods. Overnight cultures incubated at 37°C in

Levinthal's broth were diluted to produce inocula of 10^6 and 10^8 colony-forming units (CFU)/ml, verified by colony counts. All tests were carried out using brain heart infusion (BHI; Difco), with 10% Filde's medium (Difco) added and Levinthal's medium prepared from defibrinated laked horse erythrocytes as described by McLinn et al. (10). In addition, co-trimoxazole agar dilution studies and all disk diffusion tests were performed on "low-thymidine" Mueller-Hinton agar (MH; Difco) with 5% defibrinated lysed horse blood plus $2.5\ \mu\text{g}$ of reduced beta-nicotinamide adenine dinucleotide (Sigma; 7) per ml and diagnostic susceptibility test (DST; Oxoid) agar with 5% defibrinated lysed horse blood added (9). Both media were incubated overnight (37°C) to neutralize TMP-SMZ-antagonizing substances by ensuring complete phosphorylysis (2, 3). Disks, stored at -20°C , had the following potencies (in micrograms): ampicillin, 10; penicillin, 10; chloramphenicol, 30; and co-trimoxazole, 25. Antibiotic powders were stored at 4°C under desiccation. Ampicillin (Ayerst) and K penicillin G (Lilly) were adjusted to 100% potency and dissolved in sterile distilled water. Chloramphenicol (Parke-Davis) was dissolved in absolute ethanol, sulfamethoxazole (Roche) in 0.1 N NaOH, and trimethoprim (Roche) in 0.1 N lactic acid. Fresh stock solutions of antibiotics were made for each experiment.

Two concentrations of bacteria (10^6 and 10^8 CFU/ml) were inoculated onto the surface of the agar with a cotton swab to produce a uniform growth across the plate. After drying for 20 min, disks were placed onto the agar surface and the plates were

incubated at 37 C for 18 to 24 h. Zone diameters were measured to the nearest 0.5 mm by means of calipers. For the broth and agar dilution studies, serial twofold dilutions were made so that end concentrations yielded 20 to 0.078 $\mu\text{g/ml}$ for ampicillin, penicillin, chloramphenicol, and trimethoprim and 400 to 0.78 $\mu\text{g/ml}$ for sulfamethoxazole. Co-trimoxazole was tested in a 1:20 ratio of trimethoprim/sulfamethoxazole.

Broth dilution tests were carried out in a microtiter by serially diluting the antibiotic in 0.05 ml of broth through the first 11 wells; well 12 contained only broth and bacteria. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic inhibiting visual growth. Agar dilution was performed employing the Steer's replicator, delivering approximately 0.007 ml of a 10^6 or 10^9 CFU/ml suspension onto the surface of the agar (13); MIC was defined as the lowest concentration of antibiotic completely inhibiting growth. Known resistant and susceptible strains of *H. influenzae* were used as controls. Tests were considered valid if the zone sizes varied not more than ± 2 mm and the MICs varied by \pm one dilution.

RESULTS

Two of 223 *H. influenzae* tested were resistant to ampicillin (MIC ≥ 6 $\mu\text{g/ml}$). Three of 72

isolates were resistant to penicillin (MIC ≥ 1 $\mu\text{g/ml}$), and all were susceptible to chloramphenicol.

The results of the disk diffusion susceptibility tests are illustrated in Tables 1 and 2. All isolates were susceptible to chloramphenicol on all media tested at both inocula (10^6 and 10^8 bacteria/ml). Using a disk zone diameter of ≥ 22 mm as indicative of susceptibility to ampicillin and penicillin, there is no difference between the numbers of isolates susceptible to these antibiotics at the same inoculum on Levinthal's agar when compared to Filde's agar. Intermediate readings were more frequent (especially with penicillin) when MH or DST agar was used. Most *H. influenzae* were resistant to co-trimoxazole (zone diameter, ≥ 16 mm) when the disk diffusion test was carried out on Levinthal's or Filde's agar but were susceptible in all but one instance when tested at the same inoculum on low-thymidine MH or DST.

The effects of two inocula (10^6 and 10^8 CFU/ml) on disk diffusion results were tested on Levinthal's agar. The larger inoculum was associated with an increase in resistance of

TABLE 1. Effect of media on disk diffusion susceptibilities of 72 *H. influenzae* using an inoculum size of 10^8 CFU/ml^a

Medium	<i>H. influenzae</i> susceptibility (no.) to:											
	Co-trimoxazole (25 μg)			Ampicillin (10 μg)			Penicillin (10 μg)			Chloramphenicol (30 μg)		
	R (≤ 10) ^b	I (11-15)	S (≥ 16)	R (≤ 11)	I (12-21)	S (≥ 22)	R (≤ 11)	I (12-21)	S (≥ 22)	R (≤ 12)	I (12-17)	S (≥ 18)
Levinthal's/BHI	60	1	11	2	0	70	2	27	43	0	0	72
Filde's (10%)/BHI	65	0	7	2	0	70	2	25	45	0	0	72
MH plus 5% lysed horse blood	1	0	71	1	3	68	1	19	52	0	0	72
DST plus 5% lysed horse blood	1	0	71	1	3	68	1	5	66	0	0	72

^a R, Resistant; I, intermediate; S, susceptible.

^b Numbers in parentheses are zone diameters (in millimeters).

TABLE 2. Effect of inoculum on disk diffusion susceptibilities of 72 *H. influenzae* using Levinthal's agar^a

Inoculum	<i>H. influenzae</i> susceptibility (no.) to:											
	Co-trimoxazole (25 μg)			Ampicillin (10 μg)			Penicillin (10 μg)			Chloramphenicol (30 μg)		
	R (≤ 10) ^b	I (11-15)	S (≥ 16)	R (≤ 11)	I (12-21)	S (≥ 22)	R (≤ 11)	I (12-21)	S (≥ 22)	R (≤ 12)	I (13-17)	S (≥ 18)
10^6	42	2	28	2	0	70	2	1	69	0	0	72
10^8	60	1	11	2	0	70	2	27	43	0	0	72

^{a, b} See footnotes to Table 1.

H. influenzae to co-trimoxazole and an increase of intermediate readings for penicillin. Ampicillin and chloramphenicol disk diffusion susceptibilities were not affected by these inocula changes. Thus, both the inoculum size and type of agar are critical factors affecting the disk diffusion susceptibilities of *H. influenzae* to co-trimoxazole. These effects were not evident for chloramphenicol or ampicillin and did not affect the resistance of these bacteria to penicillin.

An inoculum effect was demonstrable for penicillin, ampicillin, and co-trimoxazole tested in Levinthal's and Filde's media by the broth and agar dilution methods. Figure 1 illustrates this effect for co-trimoxazole tested in Levinthal's broth at inocula of 10^6 and 10^9 bacteria/ml. The same effects on penicillin and ampicillin susceptibilities on Filde's agar are demonstrated in Fig. 2. The inoculum did not affect chloramphenicol susceptibilities in the broth or agar dilution studies. As with the disk diffusion studies, co-trimoxazole resistance was increased with the larger inoculum in broth and agar dilution studies. The effect was also present for penicillin and ampicillin, but not for chloramphenicol.

The effects of media were compared by the broth and agar dilution methods. End points were difficult to determine accurately in both Levinthal's and Filde's broth (also noted in the inoculum studies reported above). This was most marked with the microtiter method. Agar dilution tests were easiest to read with Filde's broth. MICs were slightly higher on Levinthal's agar, especially for penicillin. The results for chloramphenicol, ampicillin, and penicillin are illustrated in Fig. 3 on both Filde's and Levin-

thal's agar. In general, ampicillin and chloramphenicol demonstrated similar results by agar dilution with both media. However, penicillin was considerably less active when tested on Levinthal's medium.

The most difficult drug to evaluate in vitro was co-trimoxazole. In the initial experiments, a marked inoculum effect was noted for broth and agar dilution tests carried out on both Filde's and Levinthal's media. Most strains were resistant, and there was poor correlation between the disk diffusion and agar dilution tests. To further define this, four media were compared by the agar dilution method using an inoculum of 10^6 CFU/ml. These results are illustrated in Fig. 4 and demonstrate a significant effect of the testing medium. Most strains of *Haemophilus* ($>90\%$) were susceptible to co-trimoxazole using DST agar with 5% defibrinated lysed horse blood added, but appeared resistant when tested with the three other media, i.e., agar with 5% defibrinated lysed horse blood, BHI agar with 10% Filde's, and BHI agar with defibrinated laked horse erythrocyte filtrate (Levinthal's). MIC end points (defined as complete inhibition of growth) were very clear with DST agar, whereas trailing end points were often encountered with the other three media and a great deal of subjectivity was present in reading these tests.

DISCUSSION

The technical aspects of antimicrobial susceptibility testing of *H. influenzae* have been studied for four antimicrobials. Results indicate that ampicillin resistance ($\geq 6 \mu\text{g/ml}$) is present in 0.9% of *H. influenzae* isolated from ill children in Montreal. The predominant type of

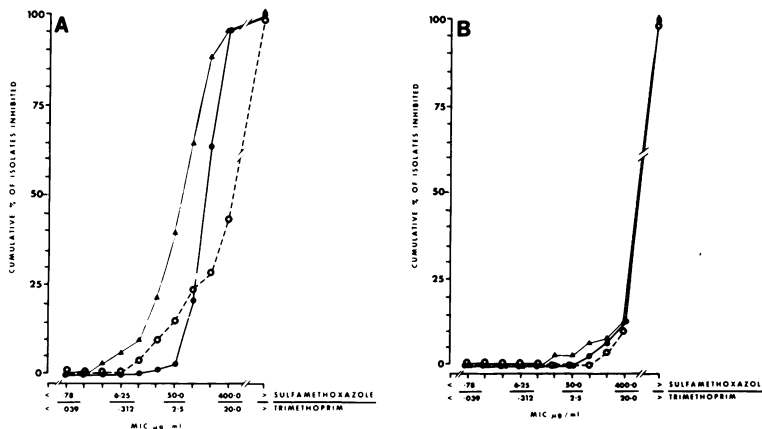


FIG. 1. Susceptibilities of 72 isolates of *H. influenzae* to trimethoprim and sulfamethoxazole alone, and in combination, tested in Levinthal's broth. (A) Inoculum, 10^6 CFU/ml; (B) inoculum, 10^9 CFU/ml. Symbols: ○ trimethoprim; ● sulfamethoxazole; ▲ trimethoprim/sulfamethoxazole.

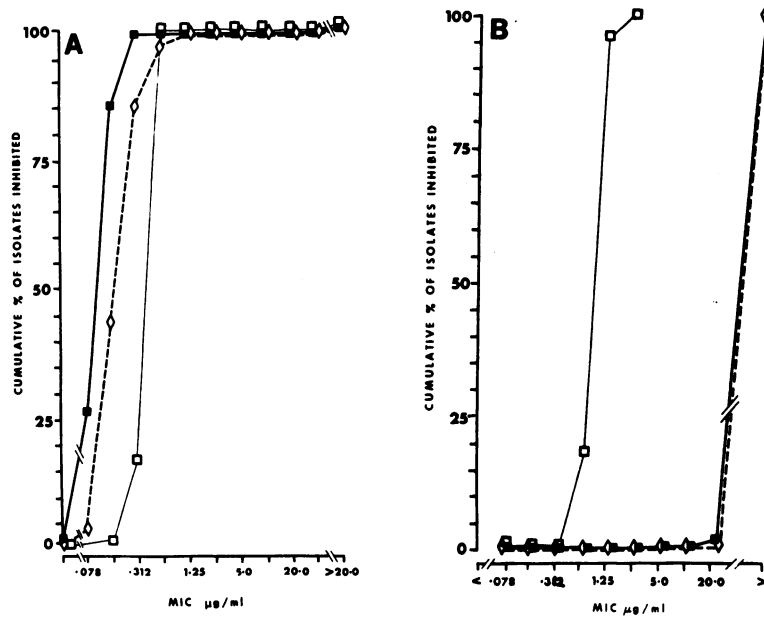


FIG. 2. Susceptibilities of 72 isolates of *H. influenzae* to ampicillin, penicillin, and chloramphenicol tested on BHI agar with 10% Filde's. (A) Inoculum, 10^6 CFU/ml; (B) inoculum, 10^8 CFU/ml. Symbols: \diamond , penicillin; \blacksquare , ampicillin; \square , chloramphenicol.

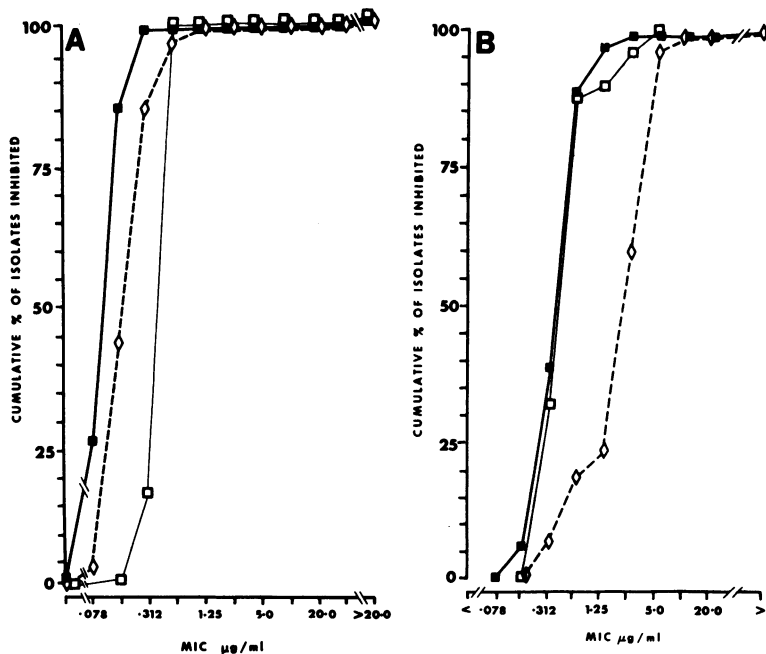


FIG. 3. Susceptibilities of 72 isolates of *H. influenzae* to ampicillin, penicillin, and chloramphenicol tested on (A) BHI agar with 10% Filde's agar and (B) Levinthal's agar. Inoculum, 10^6 CFU/ml. Symbols: \diamond , penicillin; \blacksquare , ampicillin; \square , chloramphenicol.

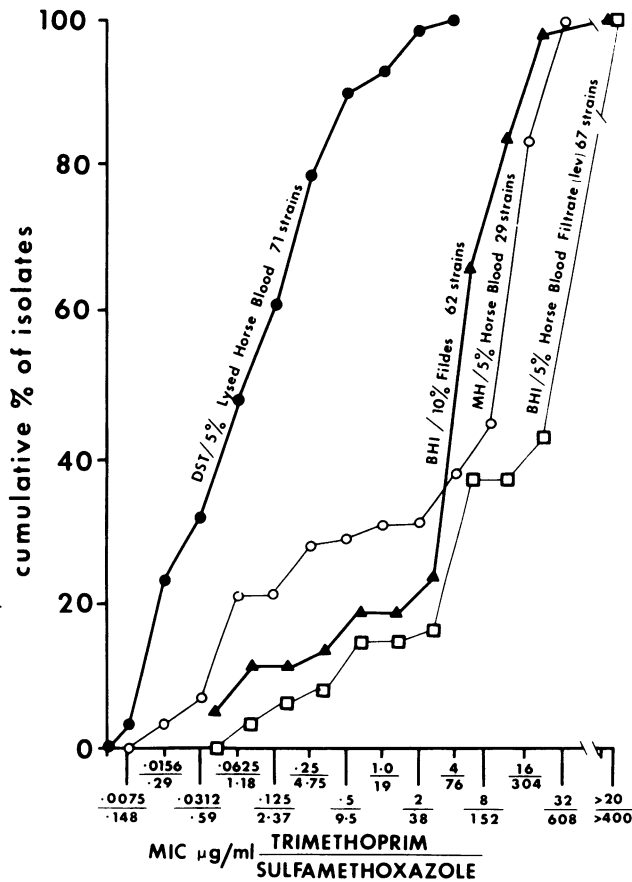


FIG. 4. Effect of four media on the agar dilution susceptibilities of *H. influenzae* to co-trimoxazole. Inoculum, 10^6 CFU/ml.

Haemophilus tested was B; however, all types were susceptible to the same effects of inocula and media. Susceptibilities of these bacteria to chloramphenicol, ampicillin, and penicillin confirm observations previously made by other investigators (4, 14, 17). No strains resistant to chloramphenicol have been encountered, and, in general, penicillin susceptibility paralleled ampicillin susceptibility.

Medium and inoculum size are most critical for *H. influenzae* susceptibilities to co-trimoxazole tested by the disk diffusion and broth/agar dilution methods. The effects of the inoculum on the quantitative susceptibility tests (broth and agar dilution) with ampicillin and penicillin are also important when interpreting these data in the light of in vivo activity of these drugs. Chloramphenicol susceptibilities were not influenced by either the inoculum or medium in these studies. As the Center for Disease Control, Atlanta (CDC Morbid. Mortal. Week. Rep., vol. 23, 1974), recommends an

inoculum size of 10^8 CFU/ml for disk diffusion susceptibility testing, this was compared to 10^6 CFU/ml for these tests. The higher inoculum (10^9 CFU/ml) used in the broth and agar dilution studies represents an undiluted 18-h broth culture.

The importance of the media in susceptibility testing to co-trimoxazole was most impressive. Conflicting data are present in the literature with regard to susceptibility of *H. influenzae* to this combination, and this most likely reflects the differences in testing techniques (7, 12, 17). The inhibitory effect of substances in the agar on sulfonamide activity was noted by Harper and Cawston (5) and Walker et al. (16). This is usually attributed to *para*-aminobenzoic acid, which inhibits sulfonamide activity, and thymidine, which inhibits trimethoprim activity. These effects can be neutralized by lysed horse blood. There is a limitation, however, on the capacity of horse blood to neutralize these inhibitors, and this is probably related to the

activity of various enzymes in horse blood, including thymidine phosphorylase (1). This is evident from the comparative evaluation of four media by the agar dilution method in our studies. The agar with the lowest content of thymidine (DST agar) demonstrated almost uniform susceptibility for the *H. influenzae* strains tested, whereas media containing higher concentrations of thymidine (including low-thymidine MH agar) demonstrated almost uniform resistance.

The technical aspects of *H. influenzae* antimicrobial susceptibility testing are complex and need further evaluation in experimental models and other in vivo situations to select the media most appropriate for predicting clinical therapeutic activities. Until these studies are available, a note of caution is urged, since it is quite obvious that the inocula and/or the media, particularly in the case of susceptibility testing to co-trimoxazole, can markedly influence the in vitro susceptibility patterns of *H. influenzae*.

The following conclusions resulted from our research.

(i) Ampicillin resistance was present in 2 of 223 clinical isolates of *H. influenzae*; 0, 3, and 1% of 72 strains tested were resistant to chloramphenicol, penicillin, and co-trimoxazole, respectively.

(ii) Resistance, as measured by the disk diffusion method, was directly related to the inoculum size and test medium for co-trimoxazole. These factors did not influence chloramphenicol or ampicillin susceptibilities but often shifted the zone diameters for penicillin to the intermediate range.

(iii) Filde's and Levinthal's media were both satisfactory for broth and agar dilution susceptibility testing with ampicillin and chloramphenicol; penicillin was less active on Levinthal's medium. Medium was critical for co-trimoxazole (see [vii] below).

(iv) Increasing the inoculum markedly increased the resistance of *H. influenzae* to co-trimoxazole; it increased resistance to ampicillin and penicillin moderately and had no effect on chloramphenicol in broth and agar dilution studies.

(v) The microtiter broth dilution test was unsatisfactory for accurate, reproducible susceptibility tests of *H. influenzae*, since clear, objective end points could not be demonstrated for any of the drugs tested.

(vi) The agar dilution method was most useful for quantitative susceptibility testing of *H. influenzae* to ampicillin, penicillin, chloramphenicol, and co-trimoxazole.

(vii) The selection of media for co-trimoxazole susceptibility testing is critical. DST agar (containing the lowest concentration of thymidine, 0.04 $\mu\text{g/ml}$) yielded clear end points in the agar dilution test and demonstrated >90% of strains to be susceptible to $\leq 1/20$ μg of trimethoprim/sulfamethoxazole per ml. End points were unclear and poorly reproducible with the other three media tested, and <25% of strains appeared to be susceptible at the same concentration of trimethoprim/sulfamethoxazole.

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LITERATURE CITED

1. Bushby, S. R. M. 1969. Combined antibacterial action in vitro of trimethoprim and sulfphonamides: the in vitro nature of synergy. *Postgrad. Med. J.* 45(Suppl): 10-18.
2. Bushby, S. R. M. 1973. Trimethoprim-sulfamethoxazole: in vitro microbiological aspects. *J. Infect. Dis.* 128(Suppl):447-462.
3. Ferone, R., S. R. M. Bushby, J. J. Burchall, W. D. Moore, and D. Smith. 1975. Identification of Harper-Cawston factor as thymidine phosphorylase and removal from media of substances interfering with susceptibility testing to sulfonamides and diamino-pyrimidines. *Antimicrob. Agents Chemother.* 7:91-98.
4. Gordon, R. C., T. R. Thompson, L. I. Stevens, and W. H. Carlson. 1974. In vitro susceptibility of *Haemophilus influenzae* to eight antibiotics. *Antimicrob. Agents Chemother.* 6:114-115.
5. Harper, G. J., and W. C. Cawston. 1945. The in vitro determination of the sulphonamide sensitivity of bacteria. *J. Pathol.* 57:59-66.
6. Khan, W., S. Ross, W. Rodriguez, G. Controni, and A. K. Szaz. 1974. *Haemophilus influenzae* type B resistant to ampicillin. *J. Am. Med. Assoc.* 229: 298-301.
7. Kirven, L. A., and C. Thornsberry. 1974. In vitro susceptibility of *Haemophilus influenzae* to trimethoprim-sulfamethoxazole. *Antimicrob. Agents Chemother.* 6:869-870.
8. Kraut, M. S., H. R. Attebery, S. M. Finegold, and V. L. Sutter. 1972. Detection of *Hemophilus aphrophilus* in the human oral flora with a selective medium. *J. Infect. Dis.* 126:189-192.
9. Leers, W. D. 1975. In vitro sensitivity of *Hemophilus influenzae* and *Streptococcus pyogenes* to co-trimoxazole. *Can. Med. Assoc. J.* 112:59S-63S.
10. McLinn, S. E., J. D. Nelson, and K. C. Haltalin. 1970. Antimicrobial susceptibility of *Hemophilus influenzae*. *Pediatrics* 45:827-838.
11. Schiffer, M. S., J. MacLowry, R. Schneerson, and J. B. Robbins. 1974. Clinical bacteriological, and immunological characterisation of ampicillin-resistant *Haemophilus influenzae* type B. *Lancet* 2:257-259.
12. Sell, S. H. W. 1967. In vitro sensitivity studies of *Hemophilus influenzae*-typable and non-typable strains. *Pediatrics* 39:214-219.
13. Steers, E., E. L. Foltz, and B. S. Graves. 1959. An

- inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. *Antibiot. Chemother.* 9:307-311.
14. Thornsberry, C., and L. A. Kirven. 1974. Antimicrobial susceptibility of *Haemophilus influenzae*. *Antimicrob. Agents Chemother.* 6:620-624.
 15. Tomeh, O. M., S. E. Starr, J. E. McGowan, Jr., P. M. Terry, and A. J. Nahmias. 1974. Ampicillin-resistant *Haemophilus influenzae* type B infection. *J. Am. Med. Assoc.* 229:295-297.
 16. Walker, N., R. P. Philip, M. W. Smyth, and J. W. McLeod. 1947. Observations on the prevention of bacterial growth by sulphonamides, with special reference to the Harper and Cawston effect. *J. Pathol. Bacteriol.* 59:631-645.
 17. Williams, J. D., and J. Andrews. 1974. Sensitivity of *Haemophilus influenzae* to antibiotics. *Br. Med. J.* 1:134-137.