Influence of Growth Medium and Supplement on Growth of *Haemophilus influenzae* and on Antibacterial Activity of Several Antibiotics

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In the present study, five non- β -lactamase- and five β -lactamase-producing strains of *Haemophilus influenzae* were used to determine whether three different growth media, Mueller-Hinton broth and agar, brain heart infusion broth and agar, and tryptic soy broth and agar, and their added supplements (0.2% hemin-0.1% IsoVitaleX, 1% hemin-1% IsoVitaleX, 2% sheep blood, 10% Fildes enrichment, 5% Fildes enrichment, 1% supplement B, 5% horse erythrocytes, and 2% hemoglobin-1% IsoVitaleX) would influence the growth rate of this microorganism and the antibacterial activity of eight antibiotics, including ampicillin, tetracycline, chloramphenicol, gentamicin, cefamandole, erythromycin, trimethoprim-sulfamethoxazole (TMP-SMX), and cefoperazone. The growth curve studies were carried out with an initial inoculum of 10^4 bacteria per ml, and MICs were determined with an inoculum of 5×10^5 microorganisms. Mueller-Hinton broth, brain heart infusion broth, and tryptic soy broth enriched with 5% Fildes resulted in a maximal growth of more than 10^8 CFU/ml at 24 h. When 10% Fildes or 2% sheep blood was added as enrichment to Mueller-Hinton broth, a considerable reduction in the growth rate of *H. influenzae* strains resulted ($P < 0.01$). Significant variations in MICs ($P < 0.01$) were observed with chloramphenicol, TMP-SMX, erythromycin, and cefoperazone when brain heart infusion agar, Mueller-Hinton agar, or tryptic soy agar was used. Chloramphenicol, gentamicin, erythromycin, and TMP-SMX were all affected by the different enrichments added to Mueller-Hinton agar. MICs were in general higher with 5% Fildes enrichment and lower with 1% supplement B. Cefoperazone was the only drug which exhibited a lower MIC in 5% Fildes enrichment for ampicillin-resistant *H. influenzae* strains.

In these days of automated microbiology, *Haemophilus influenzae* is still a difficult bacterium to grow, and automated systems have not yet been very successful in providing adequate identification and antibiotic susceptibility results. Much still remains to be discovered about the influence of different media on the growth rate of *H. influenzae* and their influence on antibiotic susceptibility data. More than 30 liquid or solid media have been used to either improve the growth of this microorganism or determine its susceptibility to antibiotics (Table 1). For example, Evans and Smith (12) noted that some agars and peptones could inhibit the growth of *H. influenzae* and hence suggested the use of a medium in which only essential growth factors were added to brain heart infusion broth. Others, like Jorgensen and Jones (22), recommended the use of Mueller-Hinton broth supplemented with 5% supplement C (Difco Laboratories, Detroit, Mich.). On the other hand, Washington et al. (50) showed that supplement C modified the susceptibility of *H. influenzae* to antibiotics and gave false resistance to ampicillin. In fact, it is very difficult to evaluate the efficacy of each medium because some are very rich and others have only the minimum requirements for the growth of this microorganism.

Several factors, including the inoculum used and the nature of the different media used, may influence the in vitro susceptibility of *H. influenzae* to antibiotics (1-3, 7, 9, 10, 14, 16, 21-23, 29, 31, 36-39, 42, 45-50, 52).

The purpose of this paper is to evaluate the influence of different basic media and different enrichments added to

these media on the growth rate of *H. influenzae* and on the MICs of several antibiotics for either β -lactamase-producing or non- β -lactamase-producing strains of *H. influenzae*.

MATERIALS AND METHODS

Organisms. Five β -lactamase-negative and five β -lactamase-positive *H. influenzae* strains were collected at the Centre Hospitalier de l'Université Laval. Isolates were identified as *H. influenzae* by Gram stain, typical colony morphology, requirement for hemin and NAD (X, V, and XV strips; Difco), and failure to hemolyze 10% horse erythrocytes in Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). Biotype was determined by the method of Kilian (25) with tests to detect tryptophanase (indole), urease, and ornithine decarboxylase. Pittman serotype (a through f or nonserotypable) was determined by direct slide agglutination with polyvalent and monovalent typing sera (Difco). All isolates were tested for production of β -lactamase by the nitrocefin method. Of the 10 strains, 7 were serotype B, while 3 were nonserotypable. Two strains were of biotype I, three were of biotype III, and five were of biotype V, as determined by the Kilian method (25).

Antibiotics. The antibiotics were kindly provided by the following pharmaceutical companies: cefamandole and erythromycin, Eli Lilly & Co., Indianapolis, Ind.; ampicillin, Ayerst Laboratories, Montreal, Quebec, Canada; chloramphenicol, Parke, Davis & Co., Montreal, Quebec, Canada; cefoperazone and tetracycline, Pfizer Inc., New York, N.Y.; trimethoprim-sulfamethoxazole (TMP-SMX), Hoffmann-La Roche, Etobicoke, Ontario, Canada; and gentamicin, Schering Corp. Ltd., Pointe-Claire, Quebec, Canada.

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TABLE 1. Media for growth of *H. influenzae*

Medium	Reference(s)
Liquids	
MHB + 10% Fildes.....	47, 48, 52
MHB + 5% Fildes.....	11, 46
MHB + 5% Fildes + 1% supplement C.....	42
MHB + 5% supplement C.....	23, 32
MHB + 2.5% supplement C.....	22
MHB + 2% hemoglobin + 1% IsoVitaleX.....	11
MHB + 1% supplement B.....	35
MHB + 5% horse blood (lysed) + 0.25% β -NAD.....	27
BHIB + 5% Fildes.....	17, 22
BHIB + 2.5% supplement C.....	19
BHIB + 1/1,000 (1 mg of β -NAD per ml + 33% defibrinated horse blood).....	36
BHIB + 1/300 (1 mg of β -NAD per ml + 33% defibrinated horse blood).....	43
BHIB + 5% horse erythrocytes.....	23, 31, 40
BHIB + 2% defibrinated sheep blood.....	49
BHIB + 1% hemin + 0.2% β -NAD.....	41
BHIB + 1% hemin + 2% IsoVitaleX + 1% L-histidine.....	
BHIB + 0.8% hemin + 0.5% β -NAD.....	18
TSB + 10% Fildes.....	52
TSB + 5% Fildes.....	30
TSB + 5% defibrinated rabbit blood + 1% IsoVitaleX.....	24, 34
Eugon + V factor strips.....	39
Eugon + 10% Fildes.....	39
Eugon + 5% Fildes.....	48
Dextrose phosphate broth 10% (1 part Fildes + 1 part 20% yeast extract).....	44
Schaedler broth + 5% Fildes.....	47
GC broth + 1% supplement B.....	36
Solids	
MHA + 1% IsoVitaleX + 5% hemoglobin.....	11, 46; Margolis ^a
MHA + 1% IsoVitaleX + 2% hemoglobin.....	33
MHA + 1% IsoVitaleX + 1% hemoglobin.....	4, 5, 48
MHA + 1% IsoVitaleX + 5% cooked rabbit blood.....	20
MHA + 5% Fildes + 1% supplement C.....	42
MHA + 2% Fildes.....	37
MHA + 5% supplement C or horse blood.....	21
MHA + 5% sheep blood.....	11
BHIA + 5% horse erythrocytes (Levinthal agar).....	3, 16, 28, 31, 49, 51
TSA + 2% Fildes.....	52
TSA + 1% hemoglobin + 1% IsoVitaleX.....	13
GC Agar CAgar base + 2% hemoglobin + 1% IsoVitaleX.....	11, 23
ASM medium + 2% IsoVitaleX + 8% rabbit blood.....	34

^a C. Z. Margolis, J. Pediatr. 87:322-323, 1975.

Media and supplements. Growth and susceptibility were determined by using brain heart infusion agar or broth (BHIA or BHIB) (Difco), Mueller-Hinton agar or broth (MHA or MHB) (BBL), and tryptic soy agar or broth (TSA or TSB) (Difco). The supplements used were IsoVitaleX (BBL), hemin extract (Eastman Kodak Co., Rochester, N.Y.), hemoglobin (BBL), Fildes enrichment (Difco), supplement B (Difco), and sheep blood and horse erythrocytes (Institut Armand Frappier, Laval, Quebec, Canada).

Growth studies. The growth rate of the 10 strains of *H. influenzae* in three different basic media (MHB, BHIB, and TSB) enriched with 5% Fildes was compared. The influence of other enrichments, including 200 μ g of hemin per ml-0.1% IsoVitaleX, 1% hemin-1% IsoVitaleX, 2% sheep blood, 10% Fildes, 1% supplement B, 5% horse erythrocytes, or 2% hemoglobin-1% IsoVitaleX added to MHB, on the growth of *H. influenzae* was also evaluated. The organisms were

suspended in supplemented broth in a volume of 2 ml and incubated for 24 h at 37°C in ambient air. An overnight culture diluted in the same medium to obtain a concentration of 10⁴ CFU/ml was used as initial inoculum for the growth curve studies. After appropriate dilutions, colony counts were performed on chocolate agar at 0, 2, 4, 6, and 24 h.

MIC studies. The MIC studies were carried out on three solid media, including MHA, BHIA, TSA enriched with 5% Fildes, and MHA supplemented with 5% Fildes enrichment, 1% supplement B, 1% hemin-1% IsoVitaleX, or 2% hemoglobin-1% IsoVitaleX. For these studies, we compared the activity of eight antibiotics (ampicillin, tetracycline, chloramphenicol, gentamicin, cefamandole, erythromycin, TMP-SMX, and cefoperazone). As described previously (5), the diluted antibiotics were added to the liquid agar at 50°C in petri dishes, the agar was allowed to solidify, and the dishes were stored at 4°C for no more than

1 day. The final concentrations of antibiotics in the plates ranged from 0.06 to 128 $\mu\text{g/ml}$. The inoculum delivered with the Steers replicator was 10^6 CFU. The plates were then incubated at 37°C for 18 h in ambient air. The MIC was defined as the lowest concentration of antibiotic at which no growth was detectable with the naked eye. *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

Statistical analysis. Analysis of variance and covariance with repeated measures and the Duncan multiple range test was used to analyze results of growth studies (8). The Kruskal-Wallis test was used to analyze the MIC data (15).

RESULTS

Growth studies. Whether we used BHIB or TSB, the growth rates of β -lactamase-nonproducing and β -lactamase-producing strains of *H. influenzae* strains were identical. All of these media enriched with 5% Fildes resulted in a maximal growth of up to 10^8 to 10^9 bacteria per ml after an incubation of 24 h. BHIB resulted in the highest log CFU per milliliter.

Figure 1 exhibits the influence of eight enrichments added to MHB on the growth rate of ampicillin-susceptible strains. At 24 h, the mean log CFU of *H. influenzae* was significantly lower in 10% Fildes enrichment (3.47) and 2% sheep blood (5.92) ($P < 0.01$). With the other enrichments, the log colony counts at 24 h varied from 7.66 with 1% IsoVitaleX–2% hemoglobin to 8.93 with 1% supplement B.

Identical observations were made with ampicillin-resistant *H. influenzae* strains. A poor growth rate was observed with 10% Fildes enrichment (4.6 log CFU/ml at 24 h) and 2% sheep blood (5.6 log CFU/ml at 24 h; $P < 0.01$). For the other enrichments, the log CFU per milliliter varied from 7.23 with 5% horse erythrocytes to 8.35 with 1% supplement B.

MIC studies. The influence of MHA, BHIA, and TSA enriched with 5% Fildes on the MICs of the eight antibiotics studied is shown in Table 2. While most of the antibiotics were not affected by the medium, noticeable differences (more than twofold) were noted with chloramphenicol ($P < 0.01$) and TMP-SMX ($P < 0.01$) against β -lactamase-nonproducing strains of *H. influenzae* and with erythromycin ($P < 0.01$), cefoperazone ($P < 0.01$), and TMP-SMX ($P < 0.01$) against β -lactamase-producing strains.

Four supplements which resulted in good growth of both β -lactamase-positive and β -lactamase-negative *H. influenzae* strains were selected and added to MHA for the MIC determinations (Table 3). These included 5% Fildes enrichment, 1% supplement B, 1% hemin–1% IsoVitaleX, and 2% hemoglobin–1% IsoVitaleX. However, 10% Fildes enrichment and 2% sheep blood were excluded, since they resulted in poor growth of *H. influenzae*. The growth curves with 0.2% hemin–0.1% IsoVitaleX and 1% hemin–1% IsoVitaleX being almost identical, 1% hemin–1% IsoVitaleX was chosen for our study. Since there was 1.15 log CFU difference in the 24-h growth between the β -lactamase-positive and β -lactamase-negative strains of *H. influenzae* when 5% horse erythrocytes was added to MHA, this supplement was excluded.

The influence of four enrichments on the MICs of eight antibiotics for β -lactamase-nonproducing strains is summarized in Table 3. Ampicillin, tetracycline, chloramphenicol, gentamicin, TMP-SMX, and cefoperazone were all affected by the different enrichments used ($P < 0.04$). For chloramphenicol, gentamicin, erythromycin, and TMP-SMX, the MICs were in general higher with 5% Fildes enrichment and

FIG. 1. Influence of eight enrichments added to MHB on the growth rate of ampicillin-susceptible *H. influenzae* strains. Symbols: ▲, 0.1% IsoVitaleX–0.2% hemin; ●, 1% IsoVitaleX–1% hemin; ■, 1% supplement B; ○, 5% horse erythrocytes; □, 1% IsoVitaleX–2% hemoglobin; △, 5% Fildes enrichment; ◆, 10% Fildes enrichment; ◇, 2% sheep blood.

lower with 1% supplement B. Cefamandole was not greatly affected by the use of different enrichments.

For β -lactamase-producing *H. influenzae* strains, the median MICs of the antibiotics varied enormously from one enrichment to the other (Table 4). All antibiotics except tetracycline were significantly affected by the medium used ($P < 0.05$). Chloramphenicol, gentamicin, cefamandole, erythromycin, and TMP-SMX exhibited higher MICs with 5% Fildes enrichment than with other supplements. In contrast, cefoperazone exhibited the lowest MIC in 5% Fildes enrichment.

DISCUSSION

In the present investigation, the use of MHB, BHIB, or TSB enriched with 5% Fildes did not modify the growth rate of β -lactamase-producing and β -lactamase-nonproducing strains of *H. influenzae*. As previously observed (6, 9), the maximum log CFU per milliliter at 24 h was around 8 to 9. Bulger and Washington (7) noted that MHB enriched with hemin and NAD failed to yield adequate growth in a microdilution system. Robinson et al. (38) found that BHIB was superior to TSB and other media for recovery of *H. influenzae* strains from blood. In our study, β -lactamase-positive and β -lactamase-negative *H. influenzae* strains grew extremely well in all three media supplemented with 5% Fildes enrichment, but use of BHIB resulted in the highest log CFU per milliliter at 24 h. The presence of disodium phosphate as a major constituent of BHIB (26) might have facilitated the growth of *H. influenzae*. BHIB is also distinguished from other media by the absence of hydrolysate or pancreatic digest of casein. Some investigators have suggested that different lots of basic medium could affect the growth rate of *Haemophilus* species (18). In the experiments described in this paper, the same lots of the three basic media and eight enrichments were used.

In our study, two of the eight enrichments added to MHB, 10% Fildes and 2% sheep blood, significantly affected the growth rate of all *H. influenzae* strains ($P < 0.01$). The reasons for the observed limited growth in the presence of 10% Fildes enrichment are far from being elucidated, but we cannot eliminate the possibility that small amounts of V factor-inactivating enzyme were present in this concentrated

TABLE 2. Influence of different media enriched with 5% Fildes on the MICs of eight antibiotics for *H. influenzae* strains

Antibiotic	MIC ^a (μg/ml) for:					
	Non-β-lactamase-producing strains			β-Lactamase-producing strains		
	MHA	BHIA	TSA	MHA	BHIA	TSA
Ampicillin	0.5	0.12	0.5	>128	>128	>128
Tetracycline	4	2	2	4	4	4
Chloramphenicol	>128	32	16	>128	32	>128
Gentamicin	2	4	4	64	64	64
Cefamandole	0.5	0.5	0.5	64	64	64
Erythromycin	>128	>128	>128	>128	4	4
TMP-SMX	32	32	1	>128	32	1
Cefoperazone	<0.06	<0.06	<0.06	<0.06	1	0.12

^a Median value for five strains.

Fildes enrichment, thus partially limiting the availability of V factor and decreasing the growth rate of *H. influenzae* (26). As for 2% sheep blood, it is the medium of choice for studying the hemolytic reactions of streptococci. By limiting the growth of *H. haemolyticus*, it allows investigators to distinguish this latter species from hemolytic streptococci (26). The nutrition requirements of *H. haemolyticus* and *H. influenzae* being similar, 2% sheep blood did not provide optimal growth of *H. influenzae*.

The MICs of ampicillin, tetracycline, gentamicin, and cefamandole measured in BHIA, MHA, or TSA enriched with 5% Fildes were not greatly affected by these media. We observed significant variations in MICs ($P < 0.01$) of chloramphenicol and TMP-SMX for β-lactamase-nonproducing strains and in MICs of erythromycin, cefoperazone, and TMP-SMX for β-lactamase-producing strains. As observed by other investigators (42), the presence in both MHA and BHIA of *p*-aminobenzoic acid, which inhibits sulfonamide, and of thymidine, which inhibits trimethoprim activity, may explain our TMP-SMX data.

In our experiments, chloramphenicol and erythromycin seemed to be less active in MHA, while cefoperazone was less active in BHIA. MICs of cefoperazone and cefamandole were similar to those observed by Baker et al. (2) for both β-lactamase-producing and β-lactamase-nonproducing *H. influenzae* strains in MHA.

Identical observations with cefoperazone were made by Bulger and Washington (7). When measured in BHIA, our MICs of ampicillin, chloramphenicol, gentamicin, erythromycin, and tetracycline are in accordance with those of many other investigators using similar inocula (9, 16, 51).

We also analyzed the influence of four enrichments on the MICs of eight antibiotics. There are very limited data on the influence of supplements to basic media on the MICs of antibiotics for *H. influenzae*. In 1975, Marks and Weinmaster (29) studied two enrichments, 10% Fildes and 5% horse erythrocytes, but these were excluded from our study owing to the limited growth observed in their presence. Marks and Weinmaster found MICs of chloramphenicol similar to those that we found when using BHIA. In our study, MICs of antibiotics were generally higher with 5% Fildes enrichment and lower with supplement B. We do not believe that the inoculum, a misreading of the plates, or a combination of these was responsible for such a disparity between the MICs of an antibiotic with the enrichments used. These studies were done in triplicate, and the inocula were controlled. Variation in the ionic content, for example, Ca^{2+} and Mg^{2+} concentration, cannot be the cause of the great disparity observed between the antibiotics and the bacterial strains used, because the basic medium was always the same for all different enrichments. Although we did not study spheroplast formations, these forms are more frequent at a higher inocula than at the inoculum used in our experiment. Furthermore, these spheroplasts should have affected only the activity of cell wall active agents.

Overall, MHB enriched with 1% hemin-1% IsoVitalX yielded results identical to those previously reported, but for several antibiotics, changes in the basic media and their enrichments greatly affected the observed growth and the MICs. The clinical significance of such observations is hard to evaluate, but as laboratories are moving toward the use of automated systems, better standardization of media for both identification and MIC studies should be strictly controlled.

TABLE 3. Influence of different enrichments on the MICs of eight antibiotics for β-lactamase-nonproducing *H. influenzae* strains

Antibiotic	MIC ^a (μg/ml) on MHA enriched with:			
	5% Fildes	1% Supplement B	1% Hemin + 1% IsoVitalX	2% Hemoglobin + 1% IsoVitalX
Ampicillin	0.5	0.12	1.0	0.25
Tetracycline	4	2	4	1.0
Chloramphenicol	>128	<0.06	32	64
Gentamicin	2	0.5	0.5	1
Cefamandole	0.5	4	0.5	0.5
Erythromycin	>128	2	32	8
TMP-SMX	32	<0.06	0.5	0.25
Cefoperazone	<0.06	0.25	0.25	<0.06

^a Median value for five strains.TABLE 4. Influence of different enrichments on the MICs of eight antibiotics for β-lactamase-producing *H. influenzae* strains

Antibiotic	MIC ^a (μg/ml) on MHA enriched with:			
	5% Fildes	1% Supplement B	1% Hemin + 1% IsoVitalX	2% Hemoglobin + 1% IsoVitalX
Ampicillin	>128	>128	8	>128
Tetracycline	4	2	4	1.0
Chloramphenicol	>128	1	<0.06	32
Gentamicin	64	<0.06	0.5	1.0
Cefamandole	64	4	1	8
Erythromycin	>128	32	32	4
TMP-SMX	128	<0.06	8	16
Cefoperazone	<0.06	0.12	0.12	8

^a Median value for five strains.

The effects of medium on growth may partly explain the paucity of automative systems effective in the identification of *H. influenzae* and measurement of its susceptibility to antibiotics.

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