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Method for Evaluating Broth Culture Media: Application to *Haemophilus*

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A method was devised to test the growth-promoting ability of a broth medium. The "dilute to extinction" method determines the inoculum required to develop heavy turbidity in a broth with overnight incubation. A statistical method using Poisson distribution was used to show that a single *Haemophilus* cell can develop heavy turbidity in an optimal broth. The dilute to extinction method was used to evaluate the shelf life of stored media, to titrate the growth factor requirements of *Haemophilus*, and to evaluate the use of purified hemin and nicotinamide adenine dinucleotide in a broth medium for the growth of *Haemophilus*. Of the media tested, the most suitable formulation was Mueller-Hinton broth supplemented with 10 μg of hemin and 10 μg of nicotinamide adenine dinucleotide per ml. The dilute to extinction method appears to be especially useful in the development of broth media for fastidious organisms. The method could also be used to assure the quality of other broth media which are required to support the growth of small inocula in the clinical or research laboratory.

The emergence of antibiotic-resistant strains necessitates antimicrobial susceptibility testing of *Haemophilus influenzae* (1, 14). Several susceptibility testing procedures have been proposed, and a variety of media have been used (2, 4, 12, 13, 15-18, 21, 23-25). Evaluation of media to determine the size of inoculum required for growth has not been reported. Inoculum size is recognized as a significant factor in susceptibility testing (3, 4, 8, 18, 19, 21, 23). A medium which will not support the growth of a small inoculum is of questionable value. Large-inoculum requirements as well as failure of some *Haemophilus* isolates to grow in supplemented broth media prompted us to devise a method to easily quantitate the efficacy of a broth medium.

MATERIALS AND METHODS

Organisms. A total of 111 clinical isolates of *Haemophilus*, consisting of 93 *H. influenzae*, 6 *H. parainfluenzae*, 5 *H. haemolyticus*, and 7 *H. parahaemolyticus*, were used in the evaluation of the broth medium. Identification to species was based on growth factor requirements as determined by X, V, and XV disks (Difco) on Mueller-Hinton (MH) agar and by hemolysis on rabbit blood agar.

Media. MH-hemin-nicotinamide adenine dinucleotide (NAD) was composed of MH broth + 10 μg of hemin per ml + 10 μg of NAD per ml. Chocolate agar consisting of hemoglobin (5%, Difco) and supplement B (1% Difco) added to tryptic soy agar (Difco) was used to maintain stock cultures and for plate count determinations.

Hemin stock solution. A 50-mg amount of hemin (Sigma Chemical Co.) was mixed with 12.6 ml of 0.5 M Na_2HPO_4, heated to dissolve, and added to 86 ml of distilled water and 1.6 ml of 1 M KH_2PO_4 (5). This solution was autoclaved at 121°C for 15 min. The final concentration was 500 μg/ml. The sterile solution was stored at 4°C.

NAD stock solution. β-NAD (Sigma Chemical Co.) was dissolved in distilled water at a concentration of 5 mg/ml and sterilized by filtration. The sterile solution was stored at 4°C.

Evaluation of basic growth media. Four base media, brain heart infusion (Difco), Trypticase soy broth (BBL), 1% tryptose (Difco), and MH broth (Difco), were evaluated for their ability to support growth of *Haemophilus* species. Each medium was supplemented with 1% supplement B (Difco) to supply X and V factors and tested with and without 0.1% agar. Nine strains of *Haemophilus* were cultured on chocolate agar and then suspended in saline to give a turbid solution. A drop of each suspension was inoculated into 5 ml of each medium in triplicate. Growth was determined by visual turbidity after overnight incubation at 35°C.

Evaluation of media by "dilute to extinction" technique. Tenfold serial dilutions through 10^{-10} of an overnight broth culture of *Haemophilus* were made in each medium. The total viable count of the inoculum culture was determined by spread plates, using chocolate agar. The dilution tubes and spread plates were incubated at 35°C for 24 h. Media interpreted as yielding heavy growth showed easily discernible turbidity corresponding to approximately 10^8 organisms per ml. The minimum number of cells per milliliter required to yield heavy growth was calculated by

multiplying the total viable count by the highest dilution.

Titration of hemin requirement. The effect of hemin concentration on the growth of small inocula was evaluated by the dilute to extinction technique. Concentrations of hemin from 0.001 to 10 μg/ml were tested in MH + 20 μg of NAD per ml. Four *Haemophilus* strains were tested.

Titration of NAD requirement. Concentrations of NAD from 0.0001 to 10 μg/ml were added to MH + 10 μg of hemin per ml. The NAD requirement for four strains of *Haemophilus* was determined by the dilute to extinction method.

Poisson distribution. An overnight culture of *H. influenzae* H125 repeatedly yielded 1.2×10^8 organisms per ml. Based on this observation, dilutions were made to yield a final dilution containing approximately one cell per milliliter. (The starting population was concurrently enumerated by the conventional spread plate technique). Fifty 1-ml samples from the dilution estimated to contain one cell per milliliter were transferred to tubes containing 4 ml of sterile MH-hemin-NAD. The tubes were shaken, incubated overnight at 35°C, and observed for growth. The observed number of "no growth" tubes was compared to the expected number of no growth tubes estimated by the Poisson distribution (22):

$$P(x) = \frac{\mu^x}{x!} e^{-\mu}$$

where x is the number of cells in a 1-ml sample, e is the base of the natural logarithm, μ is the true number of cells per milliliter in the test solution (calculated from the plate count of the starting population), and P is the probability that a 1-ml sample will contain x cells.

RESULTS

Selection of basic growth medium. The results shown in Table 1 indicate that brain heart infusion and MH were superior to the other media. However, without the beneficial effect of agar, MH was superior to brain heart infusion and was chosen as the base for further evaluation.

Evaluation of media by the dilute to extinction method. The shelf life of MH + supplement B with and without 0.1% agar was evaluated by the dilute to extinction technique. Figure 1 depicts the beneficial effect of adding agar to supplement B-enriched MH. The medium deteriorated despite the addition of agar. In an attempt to find the cause of deterioration upon storage, additional supplement B (1%), 20 μg of hemin per ml, and 20 μg of NAD per ml were added to separate portions of 7-day-old MH + supplement B. Only the addition of hemin reduced the required inoculum to ≤10 organisms per ml in the aged MH + supplement B. Note, however, that MH plus purified hemin and NAD (MH-hemin-NAD) requires ≤10 organisms per ml to yield heavy growth even after 7 days of storage.

TABLE 1. *Comparison of basic media for the growth of Haemophilus spp.*[a]

Medium	No. positive[b]/no. tested
BHI + supplement B	4/9
BHI + supplement B + 0.1% agar	9/9
TSB + supplement B	4/9
TSB + supplement B + 0.1% agar	7/9
Tryptose + supplement B	3/9
Tryptose + supplement B + 0.1% agar	7/9
MH + supplement B	8/9
MH + supplement B + 0.1% agar	9/9

[a] *H. aegyptius*, 2; *H. haemolyticus*, 1; *H. influenzae*, 2; *H. parahaemolyticus*, 2; *H. parainfluenzae*, 2. BHI, Brain heart infusion; TSB, Trypticase soy broth.

[b] Visual turbidity resulting from growth of an inoculum of approximately 5×10^4 organisms per ml.

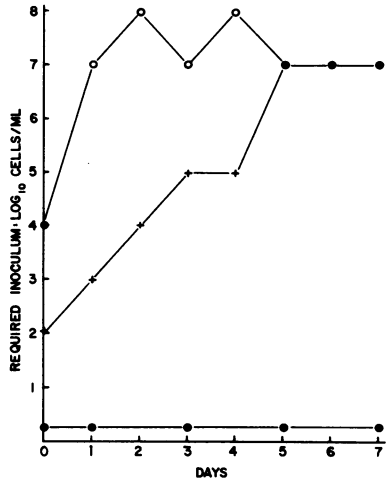

FIG. 1. *Effect of storing media (4°C, 0 to 7 days) on the size of inoculum of H. influenzae H125 required to develop heavy turbidity overnight. Media tested: MH-supplement B (○), MH-supplement B with 0.1% agar (+), and MH-hemin-NAD (●).*

The evaluation of several common sources of *Haemophilus* growth factors is shown in Table 2. The ability of media with Fildes enrichment or purified hemin and NAD to support the growth of a small inoculum proved either enrichment efficacious. The addition of the purified factors did not significantly alter the color or clarity of the medium. The ease of detecting turbidity in the clearer medium was the basis for choosing purified factors over Fildes enrichment.

Table 3 shows the results of the titration for hemin requirements of four isolates of *Haemophilus*, using the dilute to extinction method. We defined the end point as the smallest amount

of hemin which would support the growth of an inoculum of ≤10 colony-forming units/ml. *H. influenzae* H10 exhibited the largest hemin requirement, 10 μg/ml. Similarly, the concentration of NAD required by each strain was determined (Table 4). These titrations indicate that 10 μg of hemin and 10 μg of NAD per ml should be adequate for the growth of *Haemophilus* provided the hemin does not deteriorate upon storage. The lowest concentration of hemin (0.1 μg/ml) required by *H. influenzae* H125 was added to MH + 10 μg of NAD per ml and stored for 7 days. This medium still supported ≤10 colony-forming units/ml when tested by the dilute to extinction method with strain H125. MH-hemin-NAD broth stored up to 5 weeks also supported growth of ≤10 cells per ml. These results demonstrate the stability of the medium after prolonged storage.

TABLE 2. *Evaluation of some growth factor sources by the dilute to extinction method*

MH broth plus:	Growth of *H. influenzae* H125 from an inoculum (CFU/ml)[a] of:				
	10^0	10^1	10^2	10^3	10^4
Supplement B (1%, Difco)	−	−	−	−	+
Supplement C (1%, Difco)	−	−	−	+	+
Fildes enrichment (10%, Difco)	+	+	+	+	+
Hemin (10 μg/ml) + NAD (10 μg/ml)	+	+	+	+	+

[a] CFU, Colony-forming units.

TABLE 3. *Titration of hemin requirement in MH + NAD (10 μg/ml) by the dilute to extinction method*

Hemin (μg/ml)	Growth of ≤10-CFU/ml of[a]:			
	H. influenzae			*H. parainfluenzae*
	H10	H13	H125	H81
0.001	−	−	−	+
0.01	−	−	−	+
0.1	−	+	+	+
1.0	−	+	+	+
10.0	+	+	+	+

[a] CFU, Colony-forming units.

TABLE 4. *Titration of NAD requirement in MH + hemin (10 μg/ml) by the dilute to extinction method*

NAD (μg/ml)	Growth of ≤10-CFU/ml of[a]:			
	H. influenzae			*H. parainfluenzae*
	H10	H13	H125	H81
0.0001	−	−	−	−
0.001	−	−	−	−
0.01	+	+	+	−
0.1	+	+	+	−
1.0	+	+	+	+
10	+	+	+	+

[a] CFU, Colony-forming units.

Growth of *Haemophilus* in MH-hemin-NAD. One hundred eleven *Haemophilus* isolates were subcultured from chocolate agar to MH-hemin-NAD and observed for growth after overnight incubation. Growth was observed with all organisms except one strain of *H. haemolyticus*. Most cultures developed uniform turbidity, although a few organisms grew in clumps which were difficult to disperse.

MH-hemin-NAD was evaluated with 13 *H. influenzae* and 1 *H. parainfluenzae* cultures by the dilute to extinction technique. All 14 cultures yielded heavy growth in the dilution tube containing ≤10 cells per ml. The highest dilution yielding growth showed heavy growth. The dilute to extinction technique should result in some tubes receiving very few cells, suggesting that a single cell could yield heavy growth with 24 h of incubation in MH-hemin-NAD.

Poisson distribution. The Poisson equation, which predicts the distribution of particles in dilute solutions, was used to test the hypothesis that a single cell would yield heavy growth in MH-hemin-NAD.

The total viable count of the original population was determined by the spread plate technique to be 7.8×10^7 organisms per ml. The dilution blank tested ($10^{-8.0792}$) contained, on the average, 0.65 organism per ml. Solving the Poisson equation for $x = 0$, $P(0) = e^{-\mu}$, where $\mu = 0.65$, we find that the probability of having zero cells in a sample is 0.52; i.e., 52% of the 50 samples inoculated should have received no cells and result in no growth. The distribution observed resulted in 54% no growth. Solving for $x = 1$, $P(1) = 0.34$; i.e., 34% of the 50 samples should have received one cell. If two or more cells are required for growth, then the probability of no growth would be $P(0) + P(1)$, or 86% of the 50 samples. Due to the close agreement between observed and calculated results, it appears that only one organism is required to yield heavy growth in MH-hemin-NAD broth.

DISCUSSION

MH medium is unique among the four media tested in that it contains no peptone. Certain peptones inhibit *H. influenzae,* and reducing agents such as dithionate and sodium oleate may neutralize potential inhibitors (9, 11). Dubos isolated a fraction from peptone that was bacteriostatic for pneumococci only when oxidized and concluded that the redox potential of the me-

dium was critical for the initiation of growth from small inocula (7). Addition of 0.1% agar had a marked effect on the growth of *Haemophilus* in peptone-containing media but little effect with MH. Starch, present in MH broth as a "protective colloid" (20), has an effect similar to that of agar on the growth of other pathogens (6). Agar or starch may delay oxidation, neutralize inhibitors, and/or localize extracellular enzymes necessary for growth.

The dilute to extinction technique allows a quantitative evaluation of broth media and is based on the premise that an ideal medium should allow the rapid growth of small inocula—even one cell. A medium is evaluated by determining the minimum number of organisms per milliliter required to produce heavy growth overnight; the smaller the inoculum required, the better the broth. Thus, this dilute to extinction method not only can be used to compare different media as demonstrated here, but also could be used in the quality control of broth media in the clinical or research laboratory where the growth of small inocula is critical.

The Poisson equation which predicts the distribution of particles in dilute solutions was used to test the hypothesis that a single cell would yield heavy growth in MH-hemin-NAD in 24 h. Application of the Poisson equation is an excellent way to evaluate the ability of a medium to support growth. In our early attempts to demonstrate a Poisson distribution, 1-ml samples of the diluted culture were added to sterile tubes. Under these conditions inconclusive results were obtained. Parallel Poisson distribution tests were performed with 1-ml samples added to sterile tubes and 1-ml samples added to 4 ml of sterile medium. Growth in the 5-ml cultures demonstrated Poisson distribution, whereas the 1-ml cultures yielded considerably more no growth tubes than expected. Perhaps increased oxidation inhibited growth in the smaller volume. The use of the larger volume is suggested when maximum recovery is critical, such as the culture of cerebrospinal fluid.

Other investigators have reported the growth and/or antimicrobial susceptibility testing of *Haemophilus* in brain heart infusion supplemented with hemin and NAD (10, 13, 24). The minimum inoculum required to produce growth was not reported, and relatively large inocula were used. The "inoculum effect" is a major problem in susceptibility testing of *Haemophilus* (4, 8, 18, 19, 21, 23). In general, as the inoculum increases, the minimum inhibitory concentration increases. Thornsberry and Kirven (23) noted a 256-fold increase in the ampicillin minimum inhibitory concentration of a susceptible strain of *H. influenzae* when the inoculum was increased from 10^5 to 10^6 organisms per ml. Emerson et al. (8) reported a 512-fold increase in the ampicillin minimum inhibitory concentration of resistant strains of *H. influenzae* as the inoculum increased from 10^3 to 10^6 organisms per ml. Roberts et al. (21) and Bottone et al. (4) attribute these inoculum effects to the induction and growth of cell wall-defective *Haemophilus* at the higher concentrations of bacteria. Roberts et al. (21) have shown that cell wall-defective *Haemophilus* possess unusual osmotic stability. A small inoculum is required for the susceptibility testing of *Haemophilus* in order to eliminate the inoculum effect. The ability of MH-hemin-NAD to support a small inoculum seems to recommend its use in antimicrobial susceptibility testing.

The possibility of using MH-hemin-NAD broth for susceptibility testing was evaluated further. Tube dilution susceptibility tests for *Staphylococcus aureus* ATCC 25923 in MH-hemin-NAD were compared with the results obtained in Trypticase soy broth. The antibiotics tested were ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, cephalothin, and tetracycline. Minimum inhibitory and bactericidal concentration results were identical in most cases, varied by one dilution in a few instances, and only once showed a fourfold discrepancy. Moreover, results were reproducible when five strains of *H. influenzae* were tested repeatedly. MH-hemin-NAD appeared to be an excellent medium for the growth and susceptibility testing of *Haemophilus*. Other media are probably adequate, but their ability to support small inocula should be tested and considered an important parameter.

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