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Medium for Use in Antibiotic Susceptibility Testing of Anaerobic Bacteria

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A medium is described which was designed for use in testing the minimal inhibitory concentration of antibiotics for anaerobic bacteria by agar dilution. It contains: Trypticase (1%), Gelysate (1%), yeast extract (0.5%), glucose (0.1%), pyruvate (0.1%), arginine (0.1%), NaCl (0.5%), hemin (5 μg/ml), vitamin K_1 (0.5 μg/ml), agar (1.5%). The medium does not require the addition of blood to support growth of most clinical isolates of anaerobic bacteria.

At present there is no standard medium for use in testing the antibiotic susceptibility of anaerobic bacteria. Mueller-Hinton agar has been used as a standard medium for susceptibility testing of facultative bacteria for many years, but it will not support the growth of most species of anaerobic bacteria (10). A collaborative group has been working for 2 years on the development of a standard anaerobic method for determination of minimal inhibitory concentrations (MICs) of antibiotics by agar dilution. This working group consists of: V. L. Sutter (Wadsworth V.A. Hospital), W. J. Martin (UCLA), C. Thornsberry (Center for Disease Control), V. R. Dowell (Center for Disease Control), J. E. Rosenblatt (Mayo Clinic), R. J. Zabransky (Mt. Sinai, Milwaukee), and ourselves. Brucella Agar (Difco) with 5% sheep blood was suggested as a standard medium, but the group was concerned with the possible variation the blood might introduce into the test. Sheep blood varies from different suppliers, changes with age, and is easily contaminated. Brucella Agar without blood does not support growth of many anaerobes (10). We therefore undertook the development of a suitable medium that would not require the addition of blood. The medium described in Table 1 supports the growth of the anaerobes commonly isolated in clinical laboratories. The reasons for inclusion of each ingredient are given below.

Trypticase (BBL) is the best peptone that we have found for growth of anaerobes. This has also been found by others; Trypticase is the peptone component of both Schaedler medium (7) and Lombard-Dowell broth (9). We also used Trypticase because it is derived from a pure protein, casein, and should be a more consistent product than meat peptones. We also used Gelysate (BBL) peptone, which is from another pure protein, gelatin. In addition to its derivation from a pure protein, we used Gelysate because it complemented the amino acid composition of Trypticase and, in particular, was high in glycine. Glycine is fermented by some anaerobic cocci (1). Both Trypticase and Gelysate were used in 1% concentration to ensure adequate amounts of amino acids and peptides and to provide some buffering capacity.

Yeast extract was added to supply vitamins and other growth factors such as purines and pyrimidines. Several anaerobes tested, including strains of *Peptostreptococcus anaerobius* and *Bacteroides melaninogenicus*, required 0.5% and did not grow as well with 0.25%. IsoVitaleX (B-D) could not replace yeast extract; however, it may be possible to replace yeast extract at some future date with a known mixture of vitamins, purines, pyrimidines, etc.

Glucose (0.1%) was added to allow growth of the obligately saccharolytic organisms and to stimulate faster growth of most strains. We obtained faster growth of saccharolytic organisms with higher concentrations of glucose, but this resulted in a drastic drop in the pH. This was not desirable, since some antibiotics vary greatly in activity at different pH values and this could affect any slower-growing organisms on the same plate.

Arginine was added to make certain that enough would be available for *Eubacterium lentum*, which uses the arginine dihydrolase pathway (8) for energy production.

Pyruvate was added for two reasons. First, many asaccharolytic cocci, such as the *Veillonella*, utilize pyruvate as an energy source (2, 6). Second, pyruvate degrades hydrogen peroxide, forming acetate, CO_2, and H_2O. This is not nearly as fast as the reaction catalyzed by catalase, but it is more convenient to use, since pyruvate can be autoclaved with the medium.

Hemin was added to satisfy the requirements

TABLE 1. *Preparation of Wilkins-Chalgren medium*

1. Dispense the following ingredients and dissolve in 1,000 ml of water—the pH should be 7.0 to 7.2.

	Catalog no.	Company	Amt (g)
Trypticase (pancreatic digest of casein)	11921	BBL	10
Gelysate (pancreatic digest of gelatin)	11870	BBL	10
Yeast extract	0127-01	Difco	5
Glucose	D-16 73412	Fisher	1
NaCl	S-271 78440 or 78443	Fisher	5
L-Arginine—free base	A-5006	Sigma	1
Pyruvic acid—sodium salt	P-2256	Sigma	1
Agar	0140-01	Difco	15

2. Add heme and vitamin K_1 solutions to yield final concentrations of 5 μg/ml for hemin and 0.5 μg/ml for K_1 (Table 2)
3. Boil for 1 min (until agar dissolves)
4. Autoclave at 121°C for 15 min on slow exhaust aerobically
5. Cool in water bath to 50 to 55°C
6. Add antibiotic solution and dispense into petri plates

TABLE 2. *Preparation of hemin and vitamin K_1 solutions*

Vitamin K_1 stock—Add 0.2 ml of stock per liter = 0.5 μg/ml, final concentration
Sigma Chemical Co., St. Louis, catalog no. V-3501
Stock = 0.05 ml of vitamin K_1 solution + 20 ml of 95% ethanol
Filter sterilize (membrane filter)
Keep stock solution in dark bottle in refrigerator for 1 month and then discard
Hemin stock—Add 10 ml of stock per liter = 5.0 μg/ml, final concentration
Sigma Chemical Co., St. Louis, catalog no. H-2375 hemin equine type III
0.5 g of hemin + 10 ml of 1 N NaOH + 990 ml of water
Autoclave at 121°C for 12 min on fast exhaust = 500 μg/ml
Stock solution kept at room temperature under N_2 atmosphere

of *Bacteroides fragilis* (M. D. Appleman, J. J. Johnson, and T. D. Wilkins, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, I128, p. 132; 11) and other species of *Bacteroides* (5). In a commercial formulation, this could be mixed well with the other ingredients, but for making small batches we added both hemin and vitamin K_1 as a solution (Table 2) before autoclave sterilization (4). Vitamin K_1 was added, since some strains of *B. melanogenicus* require it (3). NaCl was added to make the medium isotonic so that blood could be added for use as an isolation medium when hemolysis reactions need to be detected.

Antibiotics were added (1 ml per 50 ml of medium) when the medium had been cooled to 50°C. Plates were poured immediately after addition of the antibiotic solution. The plates could be used as soon as they were dry or after storage for 1 day in either an anaerobic chamber or GasPak (BBL) type jar. Storage for a longer time would risk decay of the antibiotic in the agar, but we have not noticed any effect on growth of anaerobic bacteria.

The medium given in Table 1 has been used by seven separate laboratories to determine the MIC of 5 antibiotics on 10 standard strains of anaerobic bacteria (V. L. Sutter, A. L. Barry, W. J. Martin, J. E. Rosenblatt, V. R. Dowell, Jr., C. Thornsberry, T. D. Wilkins, and R. J. Zabransky, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 16th, Chicago, Ill., Abstr. 453, 1976). The results have been reproducible, even though the seven laboratories made the medium from the separate components and used different lots of these components.

Except for Trypticase and Gelysate peptones, the ingredients should not have to be obtained from the sources listed in Table 1, as long as good-quality products are used. However, some batches of agar from other suppliers would not remain as a liquid at 50°C, which is the temperature at which the antibiotic solution is added. Standard strains will be made available by the working group which can be used to quality control the medium composition.

The medium has been in use in our laboratory for a year and has consistently grown anaerobes as well or better than media such as Brucella Agar or Schaedler blood agar. It has the advantage of being made from relatively reproducible components and does not contain meat infusions, etc., that can vary greatly in composition.

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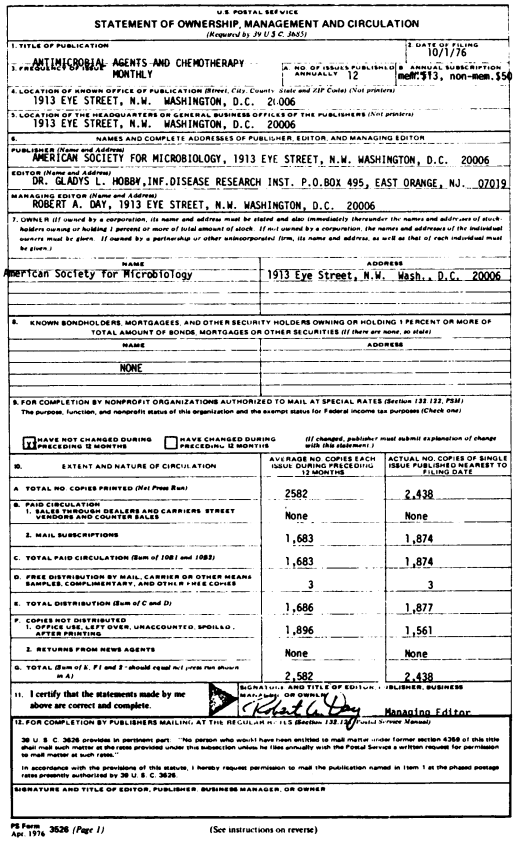

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