Selective Culture Medium to Survey the Incidence of
Haemophilus Species

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Received for publication 25 July 1969

A culture medium for the selective isolation of Haemophilus species is described. Bacitracin and nutritional supplements were incorporated in a rich basal agar medium to which rabbit blood was added to distinguish hemolytic species. Colony counts of seven typed strains of H. influenzae on this medium were within practical limits of counts on other media tested for clinical use. The bacitracin medium was as reliable as hemoglobin-agar for detecting H. influenzae and more sensitive for detecting other Haemophilus species in a clinical survey with the advantage of selectivity.

Nutritional characterization of strains. Nutritional growth requirements for X and V factors were determined by use of commercial X and V factor test discs (Difco). Determinations were confirmed by growth tests on Casman agar (Difco) prepared with yeast extract, with hemoglobin, and with both yeast extract and hemoglobin.

Media. Six culture medium formulations were utilized: (i) "Basic medium formulation" contained Casman medium sterilized and cooled to 45 C, plus 1 ml of Supplement C (Difco) and 5% (v/v) fresh rabbit blood (Robbins Laboratory, Chapel Hill, N.C.; reference 7). (ii) "Selective medium A" contained the same formulation as the basic medium plus 5 units of bacitracin (Pfizer) per ml. (iii) "Selective medium B" contained the same formulation as the basic medium plus 0.3 units of penicillin (Pfizer) per ml. (iv) "Hemoglobin-agar" contained GC Medium Base (Difco) plus 1% hemoglobin and 1% Supplement C used in broad surveys by Glezen et al. (6) and Loda et al. (8). (v) "Clear medium", essentially a modified Levinthal's medium, was prepared from Casman medium plus 1% Supplement C. This was utilized to detect iridescent colonies produced by encapsulated Haemophilus strains on a clear medium. "Plain rabbit blood-agar" was prepared by adding 5% fresh rabbit blood to sterilized Trypticase Soy Agar (BBL) cooled to 45 C. (2). This medium is used in some hospital laboratories for detection of Haemophilus species in respiratory specimens.

Quantitative media comparison procedure. The typed ATCC specimens and the two smooth colony laboratory isolates were collected from the surface of plain rabbit blood-agar and suspended in Trypticase Soy Broth. Serial 10-fold dilutions were prepared in the Trypticase Soy Broth. From each dilution, 20-uliter drops were dispensed on three plates of each test medium, with a micropipetting device (Eppendorf Micropipette, Brinkman Instruments, Westbury, N.Y.) by the procedure evaluated by Miles and Misra (9). It was essential that plates be "dried"
prior to use according to their recommendations to prevent colonies from running together. Plates were incubated for 24 hr at 37 C with 5% carbon dioxide. Colony counts were made from the dilution spotted on each plate that produced 20 to 150 colonies per spot. Colonies were counted with the aid of a dissecting microscope at a magnification of 20.

**Clinical samples.** Clinical respiratory specimens used for media comparisons were obtained from patients with cotton swabs and placed directly into 2 ml of Trypticase Soy Broth containing 1% (v/v) fresh frozen rabbit serum. The tubes were agitated at 20,000 vibrations per sec at 3 amp for 30 sec with the sterile microtip of a 75-w oscillator (Branson Instruments, Inc., Stanford, Conn.). This was predetermined to yield the highest counts of organisms from clinical specimens without producing significant loss of organisms due to rupture. Two selective medium formulations were compared with the basal medium and hemoglobin-agar in their ability to support growth from suspensions of the *H. influenzae* strains.

**RESULTS**

The *H. influenzae* ATCC stock strains harvested from plain rabbit blood-agar and diluted in Trypticase Soy Broth produced generally uniform, smooth colonies, 1 to 2 mm in diameter, on the supplemented basic medium and on the hemoglobin-agar (Fig. 1). The same suspensions usually produced only pinpoint rough colonies, 0.2 to 0.5 mm in diameter, when inoculated on the nonsupplemented plain rabbit agar medium. On the selective medium A (containing bacitracin), colonies resembled those on the basic medium (Fig. 1). On selective medium B (containing peni-

![Fig. 1. Colonies of a type b encapsulated strain of Haemophilus influenzae are shown after 24 hr of growth on Casman medium base supplemented with X and V factors and enriched with fresh rabbit blood.](image)

![Fig. 2. Colony counts on four test media of seven strains of Haemophilus influenzae.](image)
TABLE 1. Strains of *Haemophilus* organisms recovered from 100 oropharyngeal swab specimens cultured on three different medium formulations

<table>
<thead>
<tr>
<th>Species</th>
<th>Selective medium A</th>
<th>Hemoglobin-agar</th>
<th>Supplemented clear medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. influenzae</em></td>
<td>32</td>
<td>30</td>
<td>5*</td>
</tr>
<tr>
<td><em>H. parainfluenzae</em></td>
<td>30</td>
<td>9</td>
<td>6*</td>
</tr>
<tr>
<td><em>H. parahaemolyticus</em></td>
<td>27</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><em>H. haemolyticus</em></td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Low incidences were due mainly to excessive overgrowth of *Haemophilus* colonies by other species on the clear medium.

Flora. The satellite phenomenon used in some studies to aid detection of *H. influenzae* in surveys of nasopharyngeal flora (11) does not offer the advantages of a selective medium for detection of *Haemophilus* species or permit colony counts.

Penicillin was the first antibiotic incorporated into a medium for the selective isolation of *Haemophilus* species (5). In the present study, penicillin produced erratic colony sizes. This effect was not observed with media containing bacitracin. Turk (11) used a high dilution of crystal violet in a horse blood-agar medium supplemented with commercial X and V factors as an aid to carry out an epidemiological survey of type b *H. influenzae*.

Inhibition of the selected organism as well as other bacteria on a selective medium is not uncommon. In 1948, four strains of type b *H. influenzae* were reported to be sensitive to bacitracin (4). In the present study, two of the seven *Haemophilus* strains exhibited some sensitivity to the level of bacitracin used in medium A. Other workers have found respiratory strains resistant to two units of bacitracin contained in discs that they placed on plates of routine isolation media to aid the detection of *Haemophilus* colonies in mixed cultures (3). Inhibition of *Haemophilus* growth by medium A was negligible in comparison to the relative ease with which *Haemophilus* colonies could be detected on the selective medium.

Hemolytic *Haemophilus* colonies cannot be distinguished from nonhemolytic *H. influenzae* on hemoglobin-agar or on clear media. When they occur together, one or the other often goes undetected. Fresh rabbit blood included in the basic formulation overcame this difficulty. Nutritional supplementation also helped to preserve large smooth colony forms typical of encapsulated strains. From evidence obtained with the aid of this culture medium (unpublished data) and that presented by Sell et al. (10) and by Austrian (1), reason exists for greater concern about the involvement of *Haemophilus* species in respiratory illness and development of methods for their control among individuals or groups with high susceptibility.

When used together with standard media for detecting other respiratory pathogens, selective culture medium A was of value in obtaining a more complete picture of the bacterial flora in mixed specimens with greater ease and efficiency. Detection of *H. influenzae* was markedly facilitated; many strains of *H. parainfluenzae* and of hemolytic species would have otherwise gone undetected. These observations suggest that such a medium could be useful for estimating the sizes of growth of other oral species than was observed on routine blood-agar plate cultures used to detect pneumococci. Contaminating colonies of oral and respiratory species were limited to 10 to 20 colonies per plate on the selective medium and exceeded 200 to 300 on plates on the nonselective media.

The low-recovery results on the clear medium did not indicate lack of growth of *Haemophilus* bacteria but indicated an extensive overgrowth of *Haemophilus* colonies by other oral species, which made rapid recognition and pure culture isolation of *Haemophilus* species difficult or impossible. Smooth colony forms were cultivated, but these were rare and their iridescence did not measurably improve their separation from other colonies. The clear medium, like Levinthal medium, was effective in growing smooth colony strains of *Haemophilus* organisms in pure culture. Hemolytic strains could not be differentiated as independent from nonhemolytic strains, except on the selective medium containing fresh rabbit blood.

The *Haemophilus* colonies detected were predominately of the rough pinpoint varieties, typical of those common to upper respiratory passages. Serological typing could not be carried out by capsular swelling, but approximately 50% of the strains could be typed by agglutination by using a microtest to be described in another report.

**DISCUSSION**

In contrast to the oropharyngeal specimens employed in this study, most clinical specimens, such as spinal fluids, pus, etc., in which *Haemophilus* species are of greatest concern to clinicians, contain fewer contaminating oral bacteria to obscure growth of *Haemophilus* colonies on nonselective media such as hemoglobin-agar or Levinthal agar. However, on nonselective media, *Haemophilus* colonies become very difficult to detect in specimens containing a heavily mixed

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648

CRAWFORD, BARDEN, AND KIRKMAN

APPL. MICROBIOL.
hemolytic and nonhemolytic Haemophilus populations in the respiratory passages in relation to various kinds of respiratory illness, and for their qualitative detection in epidemiological investigations. The medium could be useful in evaluating the effectiveness of present and future measures to control carrier and infection rates of Haemophilus strains in specific populations with a high risk of infection.

ACKNOWLEDGMENTS

We thank Mark Johnson, Department of Biostatistics, University of North Carolina School of Public Health, for his consultation and assistance.

This investigation was supported by Public Health Service research grant DE 02352 from the National Institute of Dental Research and in part by grant FR 05333 from the Division of Research Facilities and Resources.

LITERATURE CITED