Selective Medium with DNase Test Agar and a Modified Toluidine Blue O Technique for Primary Isolation of *Branhamella catarrhalis* in Sputum

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A selective medium with DNase test agar and incorporating vancomycin (10 µg/ml), trimethoprim (8 µg/ml), and amphotericin B (2 µg/ml) supported the growth of 305 *Branhamella catarrhalis* isolates. A modified toluidine blue O technique was used after 48 h of incubation in CO₂ to overlay suspected *B. catarrhalis* colonies. A metachromatic color change was observed in 15 min, indicating DNase production. In 200 unscreened sputum samples of hospitalized patients, this method was compared with routine microbiologic procedures; 31 *B. catarrhalis* isolates were recovered with the method, compared with 22 isolated from the clinical laboratory. This medium will be particularly useful for culture of sputum, which shows inflammatory cells and gram-negative diplococci on Gram-stained smears.

*Branchamella catarrhalis* is a significant pathogen in bronchopulmonary infections and is emerging as a frequent cause of childhood otitis media (18) and sinusitis (19). In some clinical microbiology laboratories *B. catarrhalis* is rarely recovered from sputum, and only normal throat flora is reported. This may be explained in part by the difficulty in distinguishing *B. catarrhalis* colonies from *Neisseria* species, even in pure growth. Patients with underlying chronic lung disease and hospitalized elderly patients may be colonized with many different bacteria or *Candida* species, which may make the isolation of *B. catarrhalis* even more difficult.

DNase production is one of the most useful biochemical tests for the differentiation of *B. catarrhalis* from other members of the family *Neisseriaceae*, since other than *B. catarrhalis* only *Kingella denitrificans* (16) and *Neisseria* (*Moraxella, Branhamella*) caviae are able to produce DNase (4, 15). The former has been isolated from the pharynx and reported as a cause of endocarditis (9). The latter is apparently found only in guinea pigs.

A selective and differential medium would be useful to enhance the recovery of *B. catarrhalis* from specimens with mixed bacterial populations. Our objective was to develop a medium which would support the growth of *B. catarrhalis*, suppress the growth of other upper respiratory tract flora, and allow a rapid differentiation between *B. catarrhalis* and *Neisseria* spp. by detection of DNase.

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MATERIALS AND METHODS

**Bacterial strains.** *B. catarrhalis* isolates used in this study were kept frozen at −70°C in methycellulose at the East Tennessee State University Infectious Disease Laboratory. Sputum isolates were obtained from patients hospitalized at the Veterans Administration Medical Center in Johnson City, Tenn., between 1983 and 1986. The following strains were used to standardize the selective medium: *B. catarrhalis* ATCC 25238 and *Staphylococcus aureus* ATCC 25923 (positive controls for DNase production) and *Neisseria meningitidis* ATCC 13090, *Neisseria subflava* ATCC 14799, *Neisseria sicca* ATCC 9913, *Neisseria gonorrhoeae* ATCC 19424, and *Neisseria lactamica* ATCC 23970 (negative controls for DNase production).

**Culture media.** DNase test agar (Difco Laboratories, Detroit, Mich.; control no. 748061), tryptic soy agar (Difco; control no. 748518), Mueller-Hinton broths (Difco), and commercially prepared chocolate agar plates (Scott Laboratories, Fiskeville, R.I.) were utilized. In the Veterans Administration Medical Center Clinical Microbiology Laboratory, media prepared at the facility were employed.

**Standard DNase test.** Flooding of DNase test agar plates with 1 N HCl (8, 14) was used to compare our technique for DNase detection.

**Modified TBO flooding technique.** A technique suitable for mixed cultures was developed. Colonies of *B. catarrhalis* and control strains were flooded with 1 drop of buffered toluidine blue O (TBO) solution on DNase test agar after 48 h of incubation. Within 15 min the dye solution surrounding the *B. catarrhalis* colony turned to a purple magenta shade signifying DNase activity. One drop of solution placed in another area of the plate where no colonies were visible turned deep royal blue and served as a color contrast.

TBO (Fisher) with actual dye content of 57% was used. The conversion factor 100 divided by the actual dye content (20) was utilized to prepare the concentration of solutions. Distilled water was employed initially with negative results. By trial with different ionic strength buffers and pH values, 0.20 M Tris hydrochloride buffer (pH 7.8) in combination with 0.025% TBO was found to give the most consistent results. Since precipitate formation was observed, the concentration of TBO was increased to 0.04% and the buffered TBO solution was filtered with a 0.2-µm-pore-size filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.) to compensate for retention of dye aggregates in the filter membrane. This solution was used for detection of DNase production by *B. catarrhalis*.

**Comparison of the standard microbiologic techniques for sputum culture and the selective media.** Standard techniques
of sputum culture with tryptic soy agar with 5% sheep blood, chocolate agar, and MacConkey agar were compared with the selective medium by processing 200 unselected sputum specimens of hospitalized patients. The plates were inoculated and streaked in parallel. For standard techniques a complete identification was carried out for organisms present in more than 2+, using the graded system 1+ to 4+ as described in the American Society for Microbiology Cumitech 7 guidelines (3). Gram-negative bacilli and gram-positive organisms were identified with the Vitek system (Vitek Systems, Inc., Hazelwood, Mo.), and Haemophilus spp. and Streptococcus pneumoniae were identified with usual methods. Gram-negative cocci were identified as to species with the RIM-N system (Austin Biological Laboratories, Inc., Austin, Tex.). Specimens considered to be growing only normal flora in which the sputum Gram stain showed more than 25 squamous epithelial cells and fewer than 10 leukocytes per low-power field were rejected for further processing, and specimen resubmission was requested.

The selective medium plates were read at another laboratory after 48 h of incubation at 35°C under 5% CO₂. The amount of growth, Gram-stain characteristics, and results of the TBO flooding were recorded. All of the isolates that grew in more than 1+ were identified. The API 20E system (Analytab Products, Plainview, N.Y.) was used for gram-negative bacilli. Gram-negative cocci were identified by colonial morphology and pigment production (12). After subculture in chocolate agar the RIM-N system was used in the first 100 specimens and the RapID-NH system (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) was used in the second part of the study to establish the species. Diphtheroids were defined by characteristic microscopic appearance and positive catalase test. Candida species were identified with the germ-tube test.

B. catarrhalis isolates were tested for β-lactamase production with the nitrocefin disk (Cefinase; BBL Microbiology Systems, Cockeysville, Md.) after subculture on chocolate agar and retested again when reinoculated onto selective medium plates.

**RESULTS**

Initial tests revealed uniform growth of 202 isolates of B. catarrhalis with a heavy inoculum (touching confluent growth with a standard bacteriologic loop) in tryptic soy agar with 5% sheep blood supplemented with 10 µg of vancomycin per ml and 12 µg of trimethoprim per ml. To attempt additional suppression of gram-negative bacilli, the possibility of incorporating colistin was studied. Twelve recent isolates of B. catarrhalis, all β-lactamase positive, were tested for susceptibility to colistin by a standard dilution technique (13). An MIC of 0.63 to 1.25 µg/ml was found. Colistin, even at a concentration of 0.5 µg/ml, completely inhibited the growth of five B. catarrhalis isolates when added to the above medium and was eliminated from further trials.

To identify the effect of vancomycin and trimethoprim concentrations on colonial morphology, colonial diameter, and DNase production by B. catarrhalis, 12 isolates were inoculated onto Mueller-Hinton broth, incubated overnight, adjusted with broth to 0.5 McFarland standard, and serially diluted in phosphate-buffered saline (pH 7.4) to prepare a 1 to 2 CFU/µl inoculum. One hundred microliters of each isolate (100 to 200 CFU) was uniformly streaked onto DNase test agar incorporated with vancomycin (10 µg/ml) and various concentrations of trimethoprim (12, 10, 8, and 6 µg/ml), with parallel control plates of DNase test agar and chocolate. The diameter sizes of the largest B. catarrhalis colonies were similar (±2 mm) on the selective medium and chocolate and DNA agar, suggesting that there was no growth inhibition caused by the antibiotic inclusion. The amount of DNase production was also similar on DNA agar with and without antibiotics.

Amphotericin B at a concentration of 2 µg/ml was found to suppress the growth of most Candida spp. and exert no inhibitory effect on B. catarrhalis colony size or DNase production when assessed as described above.

Isolates of B. catarrhalis including the ATCC strain uniformly produced DNase, detectable by our method, when the isolated colony diameter was 2 mm or more and in confluent growth at 48 h of incubation. B. catarrhalis colonies on selective medium have a flattened creamy-white appearance, are friable, and conserve the characteristic sliding of the whole colony when pushed with an inoculation loop.

After the colonial morphology of other organisms on DNase test agar was observed, both B. catarrhalis and control strains were flooded with 1 drop of the buffered TBO solution, and the results were read at 15 min. Regarding the metachromatic color change the reaction is stable for 2 to 4 h, after which the dye slowly diffuses into the agar, losing its well-demarcated borders. The TBO flooding technique was positive for the S. aureus ATCC strain at 24 h when this strain was inoculated into DNase test agar. Control ATCC Neisseria spp. were all negative with this TBO flooding technique. No difference was observed in the color of the buffered TBO solution around colonies of DNase-negative organisms as compared with areas of the plate that had no growth.

The final formulation of the plate included DNase test agar (42 g/liter), vancomycin (10 µg/ml), trimethoprim (8 µg/ml), and amphotericin B (2 µg/ml), pH 7.26. Three hundred and five B. catarrhalis isolates were inoculated onto the selective medium (eight isolates per plate with a heavy inoculum). At 24 h 300 isolates grew. The five other isolates also grew after reinculation. The 305 isolates were tested with TBO flooding at 48 h, and the reaction was positive in all isolates.

The results of comparison between the selective medium and the routine methods in 200 unselected sputum specimens are summarized in Table 1. When the results of cultures of the nine specimens in which B. catarrhalis was found by the selective medium but not by the routine methods were
TABLE 2. Isolates and amount of growth in 200 sputum samples plated on the selective medium

<table>
<thead>
<tr>
<th>Organism (n)</th>
<th>No. of isolates with indicated amount of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram negative cocci</td>
<td></td>
</tr>
<tr>
<td>Neisseria subflava (68)</td>
<td>17 25 16 10</td>
</tr>
<tr>
<td>B. catarrhalis (31)</td>
<td>5 7 4 15</td>
</tr>
<tr>
<td>Neisseria sicca (6)</td>
<td>1 2 2 1</td>
</tr>
<tr>
<td>Neisseria mucosa (6)</td>
<td>2 2 1 1</td>
</tr>
<tr>
<td>Neisseria lactamica (3)</td>
<td>0 1 2 0</td>
</tr>
<tr>
<td>Neisseria meningitidis (2)</td>
<td>1 1 0 0</td>
</tr>
<tr>
<td>Kingella kingae (1)</td>
<td>1 0 0 0</td>
</tr>
<tr>
<td>Gram-negative bacilli</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas spp. (29)</td>
<td>7 7 3 12</td>
</tr>
<tr>
<td>Klebsiella spp. (12)</td>
<td>5 5 1 1</td>
</tr>
<tr>
<td>Enterobacter spp. (9)</td>
<td>2 4 1 2</td>
</tr>
<tr>
<td>Serratia marcescens (6)</td>
<td>3 0 2 1</td>
</tr>
<tr>
<td>Citrobacter spp. (4)</td>
<td>3 0 0 1</td>
</tr>
<tr>
<td>Proteus spp. (4)</td>
<td>1 1 0 2</td>
</tr>
<tr>
<td>E. coli (5)</td>
<td>3 0 0 0</td>
</tr>
<tr>
<td>Candida spp. (7)</td>
<td>7 0 0 0</td>
</tr>
<tr>
<td>Diphtheroids (4)</td>
<td>4 0 0 0</td>
</tr>
</tbody>
</table>

Compared, in three of the nine cases the sputum sample was rejected (poor specimen). In five cases normal throat flora and an additional organism were isolated. These organisms included Hemophilus influenzae (two isolates), S. aureus (two isolates), S. pneumoniae (one isolate), and Pseudomonas aeruginosa (one isolate).

Potential pathogens suppressed on the selective medium and recovered by routine methods in the 200 sputum samples were H. influenzae (34 isolates), S. pneumoniae (16 isolates), S. aureus (11 isolates), beta-hemolytic Streptococcus sp. (8 isolates), Haemophilus spp. (5 isolates), and Staphylococcus spp. (5 isolates).

Table 2 shows all of the significant isolates recovered on the selective medium. The TBO flooding results were positive for B. catarrhalis and for six isolates of Serratia marcescens which were recognized early as gram-negative bacilli by Gram stain.

Of 31 isolates of B. catarrhalis recovered in the selective medium, 24 (77.5%) were positive for β-lactamase production. At repeated subculture onto the selective medium, β-lactamase was consistently detected.

**DISCUSSION**

The advantages of the selective medium described herein are its transparency and a TBO technique which can be used for mixed cultures. Distinct colonies are required to observe the metachromatic change. This method could avoid the morphologic confusion between B. catarrhalis and other respiratory flora even for the inexperienced technologist. It may also take less time to presumptively identify B. catarrhalis. We emphasize that Gram staining is required before the flooding process.

In our hospitalized patients, the recovery of trimethoprim-resistant gram-negative bacilli remains a problem. With the susceptibility patterns of B. catarrhalis (1, 2, 5-7, 17), it appears difficult to find other antibiotics which completely suppress gram-negative bacilli and have no effect on B. catarrhalis. In previous work Corkill and Makin (4), using a selective medium supplemented with blood, found colistin, nalidixic acid, oxolinic acid, and aminoglycosin to be highly inhibitory against gram-negative cocci.

The principle upon which metachromatic dyes are useful for detection of DNase production are described elsewhere (10, 11, 20). The purple halo indicates the interaction of the dye with both agar and hydrolyzed DNA.

This selective medium could be used to reliably establish the prevalence of B. catarrhalis colonization in some populations and also to supplement routine techniques of sputum culture when a Gram-stained smear shows inflammatory cells and gram-negative diplococci.

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**LITERATURE CITED**


