JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1980, p. 772-775 Vol. 12, No. 6
0095-1137/80/12-0772/04$02.00/0

Comparison of Media and Culture Techniques for Detection of *Streptococcus pneumoniae* in Respiratory Secretions

TERESA C. WU,†* LINDA M. TRASK, AND ROLANDO E. PHEE
Department of Pathology and Clinical Laboratories, Mount Sinai Hospital, Hartford, Connecticut 06112

We compared the relative efficacy of three methods for the isolation of *Streptococcus pneumoniae* in lower respiratory secretions. Based on results from 294 clinical specimens, we found that *S. pneumoniae* was isolated at a frequency of 65% with 5% sheep blood agar or 5% sheep blood agar containing 5 μg of gentamicin per ml, both incubated in 5% CO_2. Anaerobic incubation of 5% sheep blood agar enhanced the recovery rate of *S. pneumoniae* to 93%. The improved efficacy with anaerobic incubation is due to the greater ease of recognition of the larger and more mucoid colonies of *S. pneumoniae*, and to the suppression of the growth of other oral bacteria present in the respiratory sections.

The isolation rate of *Streptococcus pneumoniae* in sputum cultures from patients with pneumococcal pneumonia by the standard method, i.e., 5% sheep blood agar incubated in 5% CO_2, is low. Several studies have shown that approximately 55% of patients with bacteremic pneumococcal pneumonia yield positive culture of *S. pneumoniae* from the sputum (1, 4). This low yield cannot be attributed to a decreased viability of the organism in the presence of pharyngeal flora when plating of the specimen is delayed (12). The recognition of *S. pneumoniae* is hindered by the overgrowth of other less fastidious pharyngeal organisms when nonselective media are used. Both Dilworth et al. (2) and Sondag et al. (11) reported significant improvements in the recovery of *S. pneumoniae* with 5% sheep blood agar containing 5 μg of gentamicin per ml. On the other hand, Schmid et al. (9) have concluded that gentamicin-blood agar is generally less efficient than the standard method for the isolation of *S. pneumoniae*. Howden has compared the results of aerobic and anaerobic cultivations for the primary isolation of *S. pneumoniae* from the respiratory tract of children (6). His results indicated that about half of the *S. pneumoniae* grew both aerobically and anaerobically; the remaining half grew only anaerobically. Since CO_2 was present in the anaerobic incubation only, it is not clear whether the improved recovery of *S. pneumoniae* was due to the presence of CO_2, the anaerobic environment, or both. An improved recovery rate of *S. pneumoniae* with anaerobic culture from the sputum of bacteremic pneumococcal pneumonia patients was further demonstrated by Drew (3).

† Present address: Microbiology Service, Clinical Center, National Institutes of Health, Bethesda, MD 20205.

The present report describes the results of a comparative study of the yields of *S. pneumoniae* from clinical specimens with various media and culture conditions. Our results indicate that anaerobic culture with sheep blood agar is the simplest procedure with the highest recovery of *S. pneumoniae*.

MATERIALS AND METHODS

During a 3-month period, all sputa, bronchial washings, and transtracheal aspirates submitted for routine culture to the Clinical Microbiology Laboratory of the Mount Sinai Hospital were included in the study. Specimens were first screened microscopically for the suitability for culture by the criteria and procedure outlined by Murray et al. (8). Acceptable specimens were processed for culture by the conventional protocol suggested by Isenberg et al. (7). In addition to the standard culture media, additional plates of 5% sheep blood agar and gentamicin-containing sheep blood agar were used for each specimen. Throughout the study, all platings were carried out by the same technician.

Media were prepared weekly with the aid of a Bench Top Agarmatic Sterilizer (New Brunswick Scientific Co., New Brunswick, N.J.) so that uniform depth (6 mm) was maintained for each batch of plates. Gentamicin powder was supplied by Schering Corp., Bloomfield, N.J.

The culture methods used in the present study are designated as follows: SBA-CO_2 for sheep blood agar incubated in 5% CO_2; GBA-CO_2 for sheep blood agar supplemented with 5 μg of gentamicin per ml incubated in 5% CO_2; and SBA-ANA for sheep blood agar incubated in a GasPak jar (BBL Microbiology Systems, Cockeysville, Md.). After overnight incubation (18 h) at 35°C, the plates were examined by three technicians working independently. The GBA-CO_2 plates were re-examined at the end of 48 h of incubation. To minimize the variation due to individual experience, the three examiners rotated during the 3-month study so that they worked with all three culture

methods.

Initial identification of *S. pneumoniae* was based on the typical colonial morphology seen under a dissecting microscope. Each suspect was further checked by the bile solubility and Optochin disk sensitivity tests of subcultures. In addition, representatives of each morphological type of alpha-hemolytic colonies which did not resemble those of typical pneumococci were indiscriminately subcultured and tested as described above. All subcultures were carried out on 5% sheep blood agar incubated in 5% CO_2.

All plates were reviewed by one of us (T.C.W.). Culture media giving no growth of *S. pneumoniae* were reinoculated, using colonies from plates which yielded positive results.

Quantitation of bacterial growth based on the results of standard streaking on the original plates was scored as follows: 1+, light growth in the primary streaking zone only; 2+, heavy growth in the primary streaking zone only; 3+, growth in the primary and secondary streaking zones; and 4+, growth in the primary, secondary, and tertiary streaking zones.

The differences in the recovery rates of *S. pneumoniae* by various culture media and conditions were analyzed by using Yates' correction of McNemar's test for significance (10).

An autolytic enzyme-deficient mutant, CWL-1, originally derived from nonencapsulated pneumococcal strain R36NC, was kindly provided by P. S. Vanketeschwaren of the University of Pennsylvania.

RESULTS

Of 294 acceptable specimens, there were 268 expectorated sputa, 21 bronchial washings, and 5 transtrachael aspirates. Fifty-five specimens yielded *S. pneumoniae* by one or more techniques. The yield of positive results by either SBA-CO_2 or GBA-CO_2 was similar (36 of 55 specimens, 65%); SBA-ANA gave the best yield (51 of 55 specimens, 93%). Although there is no significant difference between the results of SBA-CO_2 and GBA-CO_2, the differences between SBA-ANA and GBA-CO_2 and between SBA-ANA and SBA-CO_2 are highly significant (Table 1).

All isolates of *S. pneumoniae* were positive by the bile solubility test and produced a zone of growth inhibition of ≥15 mm with the Optochin disk sensitivity test.

The colonies of *S. pneumoniae* on the SBA-CO_2 and GBA-CO_2 plates were quite similar in morphology but different in size. Colonies were umbilicated, with a central depression, and nonmucoid in most isolates. Zones of alpha-hemolysis surrounding these colonies were characteristically large. Because of the small size of colonies on GBA-CO_2 plates, in about 2/3 of the cases the recognition of colony morphology characteristics of *S. pneumoniae* was possible only after 48 h of incubation. In contrast to the umbilicated appearance on both SBA-CO_2 and GBA-CO_2, the colonies on SBA-ANA were domed in shape, and the green hemolysis was virtually absent. A narrow green hemolysis became visible after a 30-min exposure of the plates in the air at room temperature. SBA-ANA colonies were consistently two to five times larger than their counterparts on SBA-CO_2 or GBA-CO_2 plates and had a characteristic grey, watery, or mucoid appearance. Whereas most isolates were correctly recognized by their characteristic morphologies, the identification of *S. pneumoniae* in a few instances was accomplished by indiscriminate subculturing. Of total positive results, 6 to 8% by any of these culture techniques were obtained by subculturing alpha-hemolytic colonies without the typical pneumococcal morphology.

All 55 isolates grew well by the methods of SBA-CO_2, GBA-CO_2, and SBA-ANA on subculturing despite the fact that some of the isolates failed to grow on all plates initially.

Positive specimens (34) yielded pure or nearly pure cultures of *S. pneumoniae*, varying between 1+ to 4+ in quantity, whereas the concomitant pharyngeal organisms were present in quantities of 1+ or less. The remaining 21 positive specimens consisted of a mixture of 2+ to 4+ growth of pharyngeal organisms and 1+ to 4+ growth of *S. pneumoniae*. The most common type of bacteria was viridans group streptococci followed by *Staphylococcus aureus*. Gram-negative enterics were found in only 4 cases (Table 2).

To understand the mechanisms that explain

TABLE 1. *Comparison of the number of specimens in SBA-ANA versus those in GBA-CO_2 and in SBA-ANA versus those in SBA-CO_2 showing the presence (+) or absence (−) of S. pneumoniae*[a]

SBA-ANA	SBA-CO_2		GBA-CO_2	
	−	+	−	+
−	1	3	3	1
+	18	33	16	35

[a] $P < 0.001$ for SBA-ANA and SBA-CO_2 and for SBA-ANA and GBA-CO_2.

TABLE 2. *Concomitant pharyngeal organisms with 2+ to 4+ growth from 55 S. pneumoniae positive specimens*

Organisms	No. of specimens
Viridans group streptococci	8
S. aureus	7
Proteus mirabilis	2
K. pneumoniae	1
E. coli	1
Diphtheroid	1
Neisseria sp.	1

the differences in the recovery rates and colony morphologies among the three methods, we did the following experiments. We compared the plating efficiencies of pure cultures from five clinical isolates of *S. pneumoniae* by the three methods. Results indicate that SBA-CO_2, GBA-CO_2, and SBA-ANA give essentially the same plating efficiencies in regard to the growth of *S. pneumoniae*. The ratio of average diameters of the colonies was 0.5 to 0.8:1:2 to 5 (GBA-CO_2:SBA-CO_2:SBA-ANA). It was suggested by Howden that the large mucoid colonies produced in the absence of oxygen were probably the result of the suppressed autolytic activity of *S. pneumoniae*. We repeated the plating efficiency experiment by using strain CWL-1 and found that not only were the plating efficiencies similar, but the ratios of the sizes of colonies were also the same as obtained with the clinical isolates.

DISCUSSION

Contrary to previous reports, the addition of gentamicin to sheep blood agar did not enhance the isolation rate of *S. pneumoniae* in the lower respiratory secretions. The most striking effect of gentamicin in sheep blood agar is to eliminate gentamicin-susceptible gram-negative enterics and *S. aureus* in the pharyngeal flora. Other species, such as viridans group streptococci, diphtheroids, *Neisseria* sp., and yeasts, are not affected by the presence of this antibiotic. Of 294 clinical specimens, 13% of the positive specimens had significant numbers of *S. aureus* and 7% of the positive specimens contained gram-negative enterics. The smaller colonies of *S. pneumoniae* on GBA-CO_2 plates presented difficulties in distinguishing *S. pneumoniae* from other alpha-hemolytic streptococci even after 48 h of incubation; this difficulty could account for all 19 false-negative results. In one specimen, the presence of viridans group streptococci prevented the recognition of *S. pneumoniae*, in spite of the complete elimination of *Escherichia coli* on the GBA-CO_2 plate. In view of the nature of colonized organisms in our patient populations and the difficulty with the recognition of *S. pneumoniae*, we believe GBA-CO_2 is of limited value for the primary culturing of *S. pneumoniae* from lower respiratory tract secretions.

SBA-ANA enhanced the isolation rate of *S. pneumoniae* by 27% as compared with the standard method. There were four false-negative specimens. In one case, the failure to recognize *S. pneumoniae* can be attributed to the growth of a large quantity of mucoid *Klebsiella pneumoniae*. The remaining three were due to the difficulty in distinguishing *S. pneumoniae* from other oral streptococci. In the majority of cases, the colonies of *S. pneumoniae* could be easily recognized by their larger sizes and mucoid appearance in the midst of normal flora, whereas organisms such as *S. aureus*, gram-negative enterics, diphtheroids, *Neisseria* sp., and yeasts were suppressed under the anaerobic incubation.

Strict anaerobes, which are prevalent in the mouth and upper respiratory tract as normal flora, did not interfere in the SBA-ANA method. All 294 specimens were collected and handled without taking special precautions against oxygen toxicity. In addition, SBA-ANA plates were examined after 18 h of incubation, a period too short for most fastidious anaerobes to become visible on the plates.

In spite of the fact that all 55 isolates could grow both aerobically and anaerobically on subculturing, we cannot rule out the possibility that some strains of *S. pneumoniae* may be nonaerotolerant during initial isolation. Since our initial screening focused on alpha-hemolytic colonies, it is likely that the beta-hemolytic obligatory anaerobic *S. pneumoniae* described by Yatabe et al. (13) might have been missed in our study.

S. pneumoniae is currently classified as a facultative anaerobe. This organism lacks cytochromes and utilizes oxygen through the flavoprotein system, with hydrogen peroxide as its metabolic end product. The peroxide production is not only responsible for the green discoloration surrounding colonies on blood agar, but is also in part related to the autolysis of *S. pneumoniae* (5). A reduction of hydrogen peroxide production during anaerobic growth may explain the absence of alpha-hemolysis and the absence of a central depression in the colonies. However, the reason for the formation of large mucoid pneumococcal colonies grown anaerobically is not clear. Howden emphasized the importance of the reduction in the potency of the pneumococcal autolysin in the absence of oxygen (6). We tested this possibility by employing an autolysin-deficient nonencapsulated mutant, CWL-1. As with the wild-type *S. pneumoniae*, larger colonies were produced by this mutant when grown anaerobically; however, we cannot prove that the reduction of autolysis is solely responsible for the phenomenon, and it seems more likely that a number of events could have contributed to the formation of the larger mucoid colonies in the absence of oxygen. Further studies are needed such as the rate of DNA synthesis and the activity of autolysin of *S. pneumoniae* under both aerobic and anaerobic conditions.

This study does not intend to establish the

etiological diagnosis in pneumococcal pneumonia patients, but to explore a simple, cost-effective method to improve the rate of isolation of *S. pneumoniae* in clinical laboratories. We conclude that SBA-ANA is a simple procedure with better results than the standard method and gentamicin-supplemented blood agar.

LITERATURE CITED

1. **Barrett-Connor, E.** 1971. The nonvalue of sputum culture in the diagnosis of pneumococcal pneumonia. Am. Rev. Respir. Dis. **103**:845-848.
2. **Dilworth, J. A., P. Stewart, J. M. Gwaltney, Jr., J. O. Hendley, and M. A. Sande.** 1975. Methods to improve detection of pneumococci in respiratory secretions. J. Clin. Microbiol. **2**:453-455.
3. **Drew, W. L.** 1977. Value of sputum culture in diagnosis of pneumococcal pneumonia. J. Clin. Microbiol. **6**:62-65.
4. **Fiala, M.** 1969. A study of the combined role of viruses, mycoplasmas and bacteria in adult pneumonia. Am. J. Med. Sci. **257**:44-51.
5. **Holt, L. B.** 1962. The culture of *Stretococcus pneumoniae*. J. Gen. Microbiol. **27**:327-330.
6. **Howden, R.** 1976. Use of anaerobic culture for the improved isolation of *Streptococcus pneumoniae*. J. Clin. Pathol. **29**:50-53.
7. **Isenberg, H. D., F. D. Schoenknecht, and A. von Graevenitz.** 1979. Cumitech 9, Collection and processing of bacteriological specimens. Coordinating ed., S. J. Rubin. American Society for Microbiology, Washington, D.C.
8. **Murray, P. R., and J. A. Washington II.** 1975. Microscopic and bacteriologic analysis of expectorated sputum. Mayo Clin. Proc. **50**:339-344.
9. **Schmid, R. E., J. A. Washington II, and J. P. Anhalt.** 1978. Gentamicin-blood agar for isolation of *Streptococcus pneumoniae* from respiratory secretions. J. Clin. Microbiol. **7**:426-427.
10. **Sokal R. R., and F. J. Rohlf.** 1969. Biometry, the principles & practice of statistics in biological research, p. 614. W. H. Freeman & Co., San Francisco.
11. **Sondag, J. E., R. K. Morgens, J. E. Hoppe, and J. J. Marr.** 1977. Detection of pneumococci in respiratory secretions: clinical evaluation of gentamicin-blood agar. J. Clin. Microbiol. **5**:397-400.
12. **Williams, S. G., and C. A. Kauffman.** 1978. Survival of *Streptococcus pneumoniae* in sputum from patients with pneumonia. J. Clin. Microbiol. **7**:3-5.
13. **Yatabe, J. H., K. L. Baldwin, and W. J. Martin.** 1977. Isolation of an obligately anaerobic *Streptococcus pneumoniae* from blood culture. J. Clin. Microbiol. **6**:181-182.