Epidemiological Studies of *Streptococcus pneumoniae* in Infants: Methods of Isolating Pneumococci

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A prospective study of the natural history of pneumococcal infection, which involves serial culture studies in healthy infants from 6 weeks of age onward, is in progress in our laboratory. This report describes results of a comparison of several methods for the isolation and identification of *Streptococcus pneumoniae* from the nasopharynxes and throats of these infants. Sheep blood agar, sheep blood agar with gentamicin sulfate (gentamicin agar), and mouse inoculation with 4-h broth cultures were used. Gentamicin agar proved superior to plain sheep blood agar as a solid culture medium, especially in enhancing the recovery of pneumococci from throat cultures. With gentamicin agar, similar carrier rates were found for both culture sites (nasopharynx and throat). In addition, gentamicin agar proved superior to mouse inoculation for the recovery of carrier strains from 131 nasopharyngeal culture samples processed by both methods. Sixty of 131 samples were positive for pneumococci, 25% of which would have been missed had mouse inoculation alone been used. In only three instances did we recover a strain by mouse inoculation that failed to grow on gentamicin agar; conversely, 15 strains were isolated on gentamicin agar but could not be recovered from mice. The latter observation might be explained by the fact that certain carrier strains may be relatively mouse avirulent. The use of blood agar containing gentamicin appears to offer a simple and inexpensive method for the recovery of *S. pneumoniae* and, in our opinion, provides an ideal method for the identification of pneumococcal carriers as well as for the recovery of these strains from clinical material such as sputum or ear exudates, where other and less fastidious organisms may also be present.

Pharyngeal carriage of members of *Streptococcus pneumoniae* species by healthy young children approaches 50% (3, 4, 6, 7). Variations in carrier rates may relate in part to methods used for the isolation and identification of pneumococci in cultures of the upper respiratory tract. The absence of a specific marker, such as the clear zone hemolysis of beta-hemolytic streptococci, hinders the recognition of pneumococci in cultures of the upper respiratory tract. Pneumococci do not always produce large, flat, and wet colonies with depressed centers. The less mucoid strains may be missed if present in small numbers or if crowded by less fastidious organisms commonly found in secretions of the upper respiratory tract. In addition, the colonial morphology of some strains of alpha-streptococci may simulate that of pneumococci.

Mouse inoculation of sputum cultures obtained from adults with pneumonia has been used for the isolation of pneumococci presumed

to be the etiological agent of pneumonia (1). This technique has proved sensitive and specific inasmuch as bacteria other than pneumococci commonly found in sputum contaminated with saliva are rarely pathogenic for mice. The feasibility of this technique as an epidemiological tool to survey large numbers of family members has recently been explored by Hendley and co-workers (3). Technical and logistical difficulties inherent in mouse inoculation include the time, space, and expense required to infect, segregate, and autopsy mice, particularly when large numbers of cultures are processed simultaneously. A solid medium containing a low concentration of an aminoglycoside antibiotic, such as gentamicin, appears to facilitate the recognition of pneumococci by virtue of the inhibitory effect on less fastidious bacteria such as *Neisseria* species and staphylococci, which may crowd and obscure pneumococcal colonies. However, this method has not been previously tested in a controlled fashion.

An in-depth prospective study of the natural history of pneumococcal infections has been initiated in our laboratory and remains in progress. We now report our experiences in developing suitable clinical and laboratory methods for studying the pneumococcal carrier state.

MATERIALS AND METHODS

Patient population and sampling technique. One hundred healthy term infants followed during the 1st year of life comprised this study population. Culture data now reported were based on serial cultures obtained from these infants beginning at 6 weeks of age. Nasopharyngeal (NP) and throat cultures were obtained at the time of each scheduled examination. Calcium alginate-tipped wire swabs (Inolex Corp., Chicago, Ill.) were used for obtaining NP cultures; swabs were passed along the floor of the nasopharynx and allowed to remain briefly in the posterior nasopharynx. Throat cultures were collected in the standard fashion with cotton-tipped wooden swabs.

Materials. Blood agar medium no. 2 (Inolex) was used for preparing sheep blood agar plates with and without gentamicin sulfate. Defibrinated sheep blood (Colorado Serum Co., Denver, Colo.) was added to a concentration of 5%. For gentamicin agar, gentamicin sulfate (Schering Pharmaceuticals, Kenilworth, N.J.) was added to a final concentration of 5 µg/ml. Plates were freshly prepared and used within 48 h. Blood broth cultures were prepared from Todd-Hewitt broth medium (Difco Laboratories, Detroit, Mich.) containing 5% sheep blood. Pneumococcal reference antisera, including omni-serum, pooled antisera, and individual antisera against 46 Danish serotypes, were obtained from the State Serum Institute, Copenhagen, Denmark.

Young white mice were obtained from the Animal Services Division, University of Alabama School of Medicine, Birmingham, Ala.

Culture methods. NP and throat swabs were immediately inoculated in the clinic onto sheep blood agar plates with or without gentamicin, and the inoculum was spread with a sterile wire loop. Plates were transported to the laboratory within 3 h and incubated overnight (16 to 18 h) in candle jars at 37°C.

For preparing cultures for mouse inoculation, NP and throat swabs were inoculated into blood broth and incubated at 37°C for 4 h, and 0.5 ml of this broth culture mixture was then injected intraperitoneally in mice. Mice were segregated by cage to prevent cross-infection. Animals dying within 4 days were autopsied; survivors were sacrificed at 4 days and autopsied. Specimens of peritoneal exudate and heart blood were cultured on blood agar medium with and without gentamicin sulfate.

Identification of pneumococci. Agar plates were examined with a dissecting microscope with an $\times 4$ objective to carefully search for alpha-hemolytic colonies with morphology suggestive of pneumococci. If present, large, flat mucoid colonies with depressed centers were picked for subculture. In all instances,

four alpha-hemolytic colonies from each primary culture plate were subcultured onto blood agar plates and tested for Optochin sensitivity (Optochin disk, Baltimore Biological Laboratory, Cockeysville, Md.). If two or more distinct populations with morphology suggestive of pneumococci were found, each was picked and characterized. A representative colony of the most populous alpha-hemolytic organism was routinely picked, regardless of morphology.

Those strains with an Optochin-inhibitory zone diameter of 14 mm or more were presumptively identified as pneumococci. They were further examined with reference antisera, taking colonies directly from the blood agar plate used for the Optochin sensitivity testing. Colonies were first examined with omni-serum, as recommended by Lund (5). Specimens were then tested with the nine pools of pneumococcal antisera as follows: 1 loopful each of pooled serum and test strain were thoroughly mixed on glass slides and observed for agglutination. Mixtures that yielded a homogenous milky appearance were judged negative. This method proved useful not only for determining the final battery of individual pneumococcal typing sera to be used, but it also served to confirm as pneumococci occasional strains that gave weakly positive or questionable reactions with omni-serum.

Confirmation of serotype by Neufeld reaction. The specific pneumococcal serotype was determined by the classical Neufeld test (1). Single colonies of test *S. pneumoniae* obtained from a fresh blood agar plate were mixed with a loopful of saline on a glass slide and then air-dried for 15 min. One loopful of antiserum was thoroughly mixed with the specimen, which was then covered with a glass cover slip containing 1 loopful of methylene blue. Slides were examined under oil immersion; a refractile halo surrounding the capsule was required for positive identification. Heterologous types were used as negative controls; antisera were routinely examined with stock strains of the homologous type to monitor specificity.

RESULTS

One hundred and thirty-two paired NP and throat cultures were performed. Both sites were positive in 48 cultures (36%); 7 cultures (5%) were positive only in the nasopharynx, and 3 cultures (2%) were positive only in the throat. Since most carrier infants were colonized in both the nasopharynx and throat, culturing both sites increased the pneumococcal recovery rate only slightly. The pneumococcal isolates were further characterized by serotyping. The distribution of serotypes is shown in Table 1. When pneumococci were isolated from both the nasopharynx and throat, serotypes were commonly found to be identical. In two instances, two distinct serotypes (3 and 19) were isolated from the throat, but the matching NP culture yielded only type 3.

TABLE 1. *Distribution of pneumococcal serotypes: NP and throat isolates*

Serotype no.	Source and no.	
	NP (55)	Throat (51)
23	17	14
6	15	15
3	6	5
4	4	4
1	3	3
22	3	3
48	3	3
Others ^a	4	4

^a Types 10, 11, 14, and 19.

Detection of pneumococci was compared on blood agar with and without gentamicin (Table 2). As illustrated, pneumococci were more often recovered from both the nasopharynx and the throat on the selective blood agar medium. The difference in isolate rates from throat cultures was most striking; 12 additional positive cultures resulted from the use of gentamicin agar. This represents a true increase in yield (from 19 to 31) of positive cultures from the throat of 39%.

The quantities of pneumococcal growth on sheep blood and gentamicin agar were also compared. Colony-forming units were judged as few, moderate, or numerous. Twelve of 28 NP strains isolated on both media showed greater growth on gentamicin agar, whereas only one grew more abundantly on plain sheep blood agar. Similarly, about one-half of throat strains cultivated on both media grew more abundantly on gentamicin agar, whereas only two isolates showed greater growth on plain sheep blood agar. Gentamicin agar, therefore, facilitated recovery of larger numbers of pneumococcal colonies in addition to providing a more sensitive method of detecting this bacterium.

To compare gentamicin agar with mouse inoculation for recovery of pneumococci, 131 NP specimens were processed using both methods (Table 3). Among the 60 positive pneumococcal cultures, 15 strains grew only on gentamicin agar, whereas 3 strains were recovered only from mice. Therefore, 25% of the pneumococcal strains carried in the nasopharynx would have been missed if mouse inoculation alone had been used.

DISCUSSION

The reported carriage rate of *S. pneumoniae* in the upper respiratory passages of both healthy and ill children has varied widely. In an epidemiological study performed in Syracuse, N.Y., only 4.6% of 19,462 cultures were

positive (2). At the same time, other large surveys (3, 4, 6, 7) have yielded carrier rates ranging from 40 to 60%. These differences may be explained by variations in cultivation, isolation, and identification procedures for *S. pneumoniae*.

Since comparative methodological data have been uniformly lacking in earlier investigations, we systematically and prospectively compared several different methods for detecting the pneumococcal carrier state. Our findings establish the superiority of freshly prepared gentamicin-containing sheep blood agar over plain sheep blood agar for the recovery of pneumococci from upper respiratory secretions of young infants. The inhibitory medium also proved more sensitive than mouse inoculation for recovery of carried strains. This suggests that certain strains were relatively mouse avirulent.

The use of blood agar containing gentamicin appears to offer a simple and inexpensive method for the recovery of *S. pneumoniae*, and it proved to be the optimum method for identifying pneumococcal carriers in a healthy infant population. This is especially recommended where throat cultures are obtained to document the carrier state.

Several distinct pneumococcal serotypes were encountered during this period of our study, types 23 and 6 being predominant. The

TABLE 2. *Detection of pneumococci*

Medium used	Culture site			
	NP (86 samples)		Throat (84 samples)	
	No. positive	% Positive	No. positive	% Positive
Sheep blood agar				
Without gentamicin	28	33	19	23
With gentamicin	33	38	31	37
Increased recovery with gentamicin	5	5	12	14

TABLE 3. *Comparison of gentamicin agar and mouse inoculation for the isolation of pneumococci*

Duplicate methods (131 NP cultures)	Total no. of pneumococci recovered: relation to methods			
	One method only	Each specific method	Both methods simultaneously	Either or both methods
Gentamicin agar	15	57	42	60
Mouse inoculation	3	45		

general distribution of serotypes encountered resembles the experience of others characterizing strains from children (3, 4).

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