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Pneumococci in Nasopharyngeal Samples from Filipino Children with Acute Respiratory Infections

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The presence of *Streptococcus pneumoniae* in the upper respiratory tract was studied in 318 Filipino children less than 5 years old with an acute lower respiratory tract infection. Nasopharyngeal samples were obtained from 292 children. With both quantitative bacterial culture and detection of capsular polysaccharide antigens by coagglutination, counterimmunoelectrophoresis, and latex agglutination, pneumococci were found in 160 (70%) of the 227 samples eligible for analysis. Culture was positive in 115 samples and antigen was positive in 140 samples. The culture isolation rate was significantly lower if the patient had received antimicrobial agents in the 48 h prior to the sampling. The seven most common types or groups of pneumococci were 6, 14, 19, 23, 15, 7, and 11, which together accounted for 64% of all pneumococcal findings.

There is a growing consensus that most children who die from respiratory infection in developing countries have bacterial pneumonia. Moreover, lung aspirates from patients with severe pneumonia indicate that pneumococcus and nontypeable *Haemophilus influenzae* are the most common pathogens (3, 29, 31). The rate of pneumococcal carriage in children in developing countries is extremely high, approaching 100% in several studies (10, 17, 21), while in the United States, e.g., the carriage rate in preschool children has been only 35% (12).

Much morbidity and mortality due to acute lower respiratory infections (ALRI) might be prevented if there was an effective pneumococcal vaccine available. Although the currently used polysaccharide vaccine is not sufficiently immunogenic in children less than 2 years of age, new protein-conjugated vaccines are already in the clinical development phase. Since pneumococcal vaccines are type specific, detailed epidemiological information on the types present in the target groups is necessary. It has been shown that the pneumococcal type found in the blood culture in patients with bacteremic pneumonia is usually the same as the type present in the upper respiratory tract (URT) (20). Thus, information on pneumococcal serotype distribution derived from the URT of children with ALRI has relevance for the choice of pneumococcal types for inclusion in new vaccines. There are, however, little data on the pneumococcal type distribution in the respiratory tract of healthy or ill children in developing countries (2, 20, 22).

Although bacterial culture remains the standard in clinical bacteriology, in developing countries it is not always feasible. Antigen detection methods are often relatively simple and could thus offer an alternative appropriate technology suitable for developing countries. Furthermore, antigen detection is less likely to be affected by the use of antimicrobial agents prior to the sampling (26, 32), a frequent problem in real life.

The present study was undertaken to compare the sensitivity of pneumococcal culture and with that of antigen detection methods in the same samples in a developing country setting. Nasopharyngeal aspirates (NPA) were obtained from Filipino children with ALRI. The findings were also analyzed according to whether or not the patients were given antibiotics prior to the sampling. The positive samples provided the useful information on pneumococcal type distribution.

MATERIALS AND METHODS

Patients. This prospective study was performed at the Research Institute for Tropical Medicine between 15 June and 31 October 1984. The study protocol was approved by the Internal Review Board of the Research Institute for Tropical Medicine.

The catchment area of the hospital consists of periurban slums and middle-class housing in southern metropolitan Manila, the Philippines. All patients less than 5 years old with ALRI were enrolled in the study, with parental consent. The diagnostic criteria for ALRI have been published previously (27). Relevant patient history and clinical findings were all recorded on structured case record forms. Information on the use of antimicrobial agents was based on interviews with the parents.

NPA. A catheter (Vygon) was inserted through the nostril. Suction was applied only after the catheter elicited a vigorous cough reflex, and a sample volume ranging from 0.5 to 3 ml was collected in a sterile mucus trap (Vygon). The same samples were also used for extensive virological investigations, the results of which have been published elsewhere (27).

Quantitative bacterial culture and Gram staining. Immediately after specimen collection, a portion of the NPA samples was homogenized with an equal volume of dithiothreitol solution (Sputasol) for 10 min at room temperature. A Gram-stained smear of the homogenized sample was examined by light microscopy. The bacterial findings were recorded and graded as either predominant (++) or minor (+). Polymorphonuclear leukocytes and epithelial cells were also counted to assess the purulence of sample. Samples with ≥25 polymorphonuclear leukocytes per microscopic field were classified as purulent.

One microliter each of homogenized NPA samples, undiluted or diluted 10^{-2} or 10^{-3}, was inoculated onto a set of

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TABLE 1. Pneumococcal antigen detection compared with pneumococcal culture in 227 NPA[a]

Pneumococcal antigen	Pneumococcal culture (no. of samples)		Total no. of samples
	Positive	Negative	
Negative	95	45	140
Negative	20	67	87
Total	115	112	227

[a] The agreement between the tests is moderate (K = 0.43).

three blood agar plates (without antibiotics, with gentamicin, or with bacitracin) and on MacConkey agar plates. MacConkey plates were used to find gram-negative bacteria, which sometimes may give false-positive reactions in antigen detection of pneumococci. The number of colonies on the plates after 1 to 2 days of incubation at 37°C was recorded, and the bacteria were identified by standard methods (19). The routine dilution method used in the laboratory allowed the reliable detection of isolates with $\geq 10^5$ CFU/ml.

Detection of pneumococcal capsular polysaccharide antigens. The remaining part of the NPA samples was stored at −20°C until analysis. For antigen detection, the samples were diluted 1:2.5 in phosphate-buffered saline containing 20% inactivated fetal calf serum, 2% Tween 20, and 0.01% merthiolate, sonicated for 1 to 3 min, and heated in a boiling water bath for 5 min. Three different methods were used to detect pneumococcal antigens in the heated samples. (i) Counterimmunoelectrophoresis (CIE) was performed as described earlier (18). For the primary demonstration of pneumococcal antigens, Omniserum and a mixture of eight type-specific antisera (for the presumably most common types or groups 3, 4, 6, 8, 9, 18, 19, and 23) were used. (ii) For the detection of neutral polysaccharides of types 7 and 14, type-specific latex reagents were prepared as described earlier (18). (iii) In addition, the coagglutination (COA) test (Phadebact Pneumococcus test; Pharmacia, Uppsala, Sweden) was performed to demonstrate all pneumococcal capsular polysaccharides.

When a positive finding was obtained by CIE and/or COA, the capsular polysaccharide type was further determined by CIE, using antiserum pools and the complete selection of type- or group-specific antisera. All pneumococcal antisera were purchased from Statens Serum Institut (Copenhagen, Denmark).

Statistical methods. The results were analyzed with the SAS System. Statistical significance of differences in rates was calculated by the χ^2 test. The strength of agreement between two different methods was evaluated by calculating the kappa (K) values for two-way tables (1). The K values were classified as follows: <0.20, poor; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, good; and 0.81 to 1.00, very good agreement (1).

RESULTS

Patients and samples available. A total of 318 patients with ALRI were enrolled; 135 (42%) patients were admitted to the hospital for treatment. Many patients were severely ill, and 32 patients died after admission. The distribution of the main entry criteria in the study group has been published previously (28). A definite infiltrate on the chest X ray was seen in 38% of the patients, as judged by coded readings by two pediatric radiologists. In the remaining patients, the diagnosis of lower respiratory involvement was based on clinical signs or symptoms.

Pneumococcal antigen was searched for in 292 samples (92%). These samples constitute the basis for the analysis of pneumococcal type distribution. Both antigen detection and culture results were available for 227 patients (71%), which is the subgroup for the comparison of the different methods. Both subgroups had distributions of clinical findings similar to that of the original group of enrolled patients.

Pneumococcal findings. By culture, *Streptococcus pneumoniae* was found in 115 (51%) of the 227 NPA samples. Pneumococcal antigen was detected in 140 (62%) of the same samples (Table 1). Both methods were positive in 95 (42%) samples. Twenty (17%) of the culture-positive samples were negative in the antigen detection assay. There was no correlation between negative antigen finding and number of pneumococcal colonies in culture; actually, more than 90% of the culture-positive samples grew more than 10^6 bacteria per ml. Forty-five (32%) of the 140 antigen-positive samples were negative by culture. In 17 (38%) of these samples, gram-positive diplococci were seen as the predominant finding in Gram-stained smears. Some 98.6% of the samples were considered purulent, with more than 25 polymorphonuclear leukocytes per microscope field.

Effect of antimicrobial agents. Information on the use of antimicrobial agents within 48 h prior to enrollment was available from 223 of the 227 ALRI patients; 66 (30%) patients had a history of antimicrobial use (Table 2). Pneumococcal culture was positive significantly less often in the patients with antibiotic therapy (38%) than in those who did not use antibiotics (56%; $P < 0.05$). There was no difference between the two groups in the number of colonies detected in culture. Again, more than 90% of the cultures grew more than 10^6 bacteria per ml, and in about 30% of the isolates the colonies were too numerous to count in both groups.

Antigen detection was positive equally often in both patient groups. In culture-positive samples, antigen detection was positive in 83% of specimens if the patient had not received antimicrobial agents and in 80% if the agents had been used. In

TABLE 2. Pneumococcal findings in culture and by antigen detection versus use of antibiotics within 48 h prior to nasopharyngeal sampling

Antibiotics within 48 h prior to sampling:	No. (%) of patients with given results by:				No. (%) of patients with any positive finding (+) or all tests negative (−)	
	Culture		Antigen detection			
	+	−	+	−	+	−
Yes, 66 patients (30%)	25 (38)	41 (62)	35 (53)	31 (47)	40 (61)	26 (39)
No, 157 patients (70%)	88 (56)	69 (44)	103 (66)	54 (34)	118 (75)	39 (25)
Significance	$P < 0.05$		NS[a]		$P < 0.05$	

[a] NS, not significant.

TABLE 3. Proportion of patients with preceding antimicrobial therapy in relation to antigen and culture findings[a]

Pneumococcal findings	No. of patients	% Receiving antimicrobial therapy
Antigen positive, culture positive	93	22
Antigen negative, culture positive	20	25
Antigen positive, culture negative	45	33
Antigen negative, culture negative	65	40

[a] Information on the use of antimicrobial agents was available from 223 patients.

culture-negative samples, antigen was found in 43% of those not given antibiotics and in 37% of those previously given antibiotics. An analysis of the combined findings of antigen detection and culture in relation to antimicrobial use (Table 3) showed that when both methods were positive only 22% of the patients used antimicrobial agents; when both methods were negative, 40% had used antimicrobial agents ($P < 0.05$).

Gram stain. As a standard method, Gram stain was also done, but this proved very insensitive and possibly nonspecific. Gram-positive diplococci were seen as the predominant finding in 67 of 115 (58%) of the culture-positive NPA samples, in 73 of 140 (52%) of the antigen-positive samples, and in 24 of 67 (36%) of the NPA samples with no other evidence of pneumococcal involvement.

Sensitivity of methods. In the 292 samples for which antigen detection results were available, COA was the single most sensitive method: it was positive in 144 (85%) of 170 antigen-positive samples, while CIE was positive in 128 of 170 (75%) of these samples. The two tests had a moderate level of agreement (K = 0.59). Latex agglutination was used only for the detection of pneumococcal types 7 and 14. For this purpose, it was invaluable, as only 8 (25%) of the 32 strains of these serotypes gave a definite reaction with both COA and CIE. Individually, COA detected 78% and CIE detected 38% of types 7 and 14.

Pneumococcal type distribution. In 132 of 170 (78%) of the antigen-positive samples, pneumococcal type or group could be identified by CIE or latex agglutination (Table 4). Two different antigens in the same sample were detected in samples from 14 patients (8%). Pediatric types or groups 6, 14, 19, and 23 accounted for 55% and the seven most common types or groups (6, 14, 19, 23, 15, 7, and 11) accounted for 64% of the pneumococcal types or groups identified.

TABLE 4. Pneumococcal type or group distribution in 170 Filipino children by age group[a]

Pneumococcal type	Total no. (%) found	No. found in given patient age group (mo)			
		0–11 (n = 100)[b]	12–24 (n = 111)	25–47 (n = 62)	48–60 (n = 19)
6	32 (17.4)	7	15	6	4
14	27 (14.7)	12	9	5	1
19	27 (14.7)	10	6	9	2
23	16 (8.7)	7	3	5	1
15	7 (3.8)	1	3	2	1
7	5 (2.7)	2	2	1	0
11	4 (2.2)	1	1	2	0
9, 13, 21[c]	9 (4.9)	3	4	2	0
32, 33, 35, 45[d]	8 (4.3)	2	5	1	0
3, 4, 8, 10, 16, 20, 25, 29, 31, 39, 44[e]	11 (6.0)	3	7	1	0
Nontypeable	38 (20.6)	14	15	5	4
Total no. found	184 (100)	62	70	39	13

[a] Two types were detected simultaneously in 14 children.
[b] n = number of NPA samples.
[c] Three of each type were found.
[d] Two of each type were found.
[e] One of each type was found.

DISCUSSION

We investigated the presence and type distribution of pneumococci in the URT of 318 Filipino children with ALRI, comparing immunological antigen detection methods with bacterial culture. The results demonstrate that antigen detection may be more sensitive than culture in demonstrating pneumococci in URT, particularly in children with prior antimicrobial therapy. Antigen detection is a feasible method for studies on pneumococci in developing countries.

We did not type the strains isolated in culture, but it has been demonstrated earlier in our laboratory, with the same methods as in the present study, that the typing results from antigen detection match very well those obtained from typing of cultured strains (16). In that study, 48 NPA samples from 315 children with symptoms of acute respiratory tract infection were positive for pneumococcus by both antigen detection and culture. In 45 (98%) of these, the type or group was the same by both methods, indicating that the specificity of the antigen detection tests was high (16).

The present data show that pneumococcal capsular polysaccharides are present in amounts detectable by routine immunological methods in 83% of NPA from which pneumococci can be cultured, corresponding to amounts of $\geq 10^5$/ml. When the antigen was found, it could be identified with type- or group-specific antisera in 78% of the cases. The results obtained by COA and CIE had a moderate level of agreement (K = 0.59), but CIE is clearly less useful in the detection of neutral capsular polysaccharides of types 7 and 14.

Forty percent of the NPA samples from which no pneumococci grew in culture contained pneumococcal antigen. In about 40% of these samples, gram-positive diplococci were also found as the predominant bacteria in Gram-stained smears. Several plausible reasons for the antigen-positive, culture-negative findings exist. Although culture was performed promptly after the samples were obtained, the viability of the bacteria may in some cases have been lost before culturing. Various host defense mechanisms (e.g., antibody, complement, or phagocytic activity) operating during infection may also have killed the bacteria before the samples were taken. Use of antimicrobial agents prior to the sampling obviously impairs the culture results (14). The culture isolation rate for pneumococcus was significantly lower if the patient had used antibiotics during the preceding 48 h (Table 2). In all of these cases, it is expected that bacterial antigen components can remain detectable after the bacteria are no longer viable. Indeed, our earlier studies have shown that 33% of culture-negative middle ear exudates also contain pneumococcal antigen (18).

Could the antigen-positive but culture-negative results be false-positive findings? Polysaccharides cross-reactive with certain pneumococcal types do occur, e.g., in enteric bacteria (25). *Escherichia coli* or *Klebsiella* sp. were actually isolated from 20% of the antigen-positive, culture-negative samples. However, it is unlikely that these would be the rare cross-reactive *E. coli* or *Klebsiella* capsular types among about 200 possible types. Cross-reactions are possible also with alpha-hemolytic streptococci (13, 30), but recent evidence suggests that this is important in saliva but not in nasopharyngeal samples (4). In

the present study, other respiratory pathogens and nonpathogens were isolated with comparable frequency in all four subgroups of Table 1, with *H. influenzae* being the most frequent pathogen (30 to 40% of the samples).

The value of finding pneumococcal antigen in NPA for etiological diagnosis has been the subject of much debate. Earlier, it was considered to correlate well with the clinical diagnosis of bacterial pneumonia (5, 23). More recent data suggest, however, quite the opposite in demonstrating that finding pneumococci in upper airways does not indicate pneumococcal infection in lower airways (16). On the other hand, if pneumococci are not present in NPA, pneumococcal etiology is unlikely (16), and if pneumococci are cultured from blood in patients with invasive disease, the same type is present in the URT (20).

Carriage of pneumococci is very common in children (6, 11), especially in developing countries, where the carriage rate in healthy children can be close to 100% (10, 17, 21). On the other hand, there are reports suggesting that the amount of antigen in sputum samples due to carriage is too low for immunochemical detection (4, 8). Our results indicate that at least in NPA from Filipino children the bacteria are present in sufficiently high numbers to allow antigen detection by the methods used.

Eighty-four different capsular polysaccharide types have been identified in pneumococci. However, only about 20 of them are common causative agents of pneumococcal infections (24), which makes the development of type-specific pneumococcal vaccines feasible. The situation is somewhat complicated by the fact that different capsular types may cause disease in different age groups and in different parts of the world.

In industrialized countries, pneumococcal type distribution in children has been relatively well documented (6, 7, 9, 11, 12, 24). Among 295 pneumococcal strains isolated from middle ear exudate during acute otitis media in Finnish children, the most common pneumococcal types were the pediatric types or groups 6, 14, 19, and 23, which together accounted for 72% of all cases (15).

So far, information on pneumococcal type distribution in children in developing countries is scarce. Although the pediatric types are usually well represented in samples from sick children, studies from The Gambia (31), Nigeria (22), Pakistan (20), and Papua New Guinea (2) indicate that significant geographical variation in the type distribution exists. Different types are also found in patients with different clinical diagnoses (2, 22). In the present study, the proportion of the pediatric types in the URT secretions of Filipino children with ALRI was 55%, but 21 other types were also found.

Even with the few studies available, significant differences can be identified in pneumococcal type distribution in different developing countries. These data underscore the need for more detailed epidemiological information on pneumococci, essential for the development of type-specific pneumococcal conjugate vaccines. Antigen detection is a useful tool for epidemiological surveys of types prevalent in the population.

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