Characterization of Nontypable *Streptococcus pneumoniae*-Like Organisms Isolated from Outbreaks of Conjunctivitis

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From January through June 1980 seven colleges and universities in various parts of New York State (NYS) reported outbreaks of conjunctivitis affecting at least 1,500 students of both sexes. Of the 125 conjunctival swabs tested in our laboratory, organisms identified as nontypable *Streptococcus pneumoniae* were isolated in pure culture from 24% and in combination with other organisms from 22%. Although bile-soluble and susceptible to optochin, the isolates had a dry-colony appearance and no typable capsule with the Neufeld capsular-swelling test. Mouse passage of four representative NYS isolates did not stimulate production of a typable capsule. We subsequently chose to refer to these isolates as *S. pneumoniae*-like organisms. Of primary importance to our study, all NYS isolates tested were similar in biochemical and immunological reactions, antibiotic susceptibility, and virulence in mice. Of 18 strains referred to us from three other outbreaks (California, 1980; NYS, 1981; Illinois, 1981), four of the six tested biochemically gave the same biochemical reactions as the four NYS isolates, and 16 of the 18 tested immunologically reacted strongly with antisera produced against those four isolates, showing line(s) of identity with each other and with the NYS isolates.

*Streptococcus pneumoniae* has rarely been isolated from healthy eyes, but it has been implicated in conjunctivitis in children (4, 10) and adults (5, 16), although not in neonates (17). None of these reports specify whether the conjunctival isolates are typable, and therefore no correlation between either typable pneumococci or specific pneumococcal capsular types and conjunctivitis has been established. However, a relationship between organisms identified as nontypable *S. pneumoniae* and conjunctivitis has been suggested by a retrospective study of the occurrence of capsular types of pneumococci isolated from infected foci and body fluids at Boston City Hospital during selected years between 1935 and 1974 (8). More than 21% of the *S. pneumoniae* isolated from eyes was nontypable, compared with 0 to 6% nontypable from other body sites. A low incidence of organisms identified as nontypable *S. pneumoniae* in body sites other than eyes has also been reported in a more recent study concerning types of pneumococci isolated from ordinarily sterile clinical specimens; only 2.2% of the 809 pneumococcal isolates were nontypable (6).

Nontypable *S. pneumoniae*-like organisms were recently implicated as the causative agents of outbreaks of conjunctivitis in New York State (NYS). In January 1980 the university in Canton and a college in Oneonta reported increasing numbers of cases of conjunctivitis among students. Within a few weeks the same problem was reported from colleges and universities in Albany, Buffalo, Ithaca, New Paltz, and Troy. By early June these seven institutions had reported a total of 1,567 cases of conjunctivitis among students, and organisms identified as nontypable *S. pneumoniae* had been isolated from conjunctival specimens tested throughout the state.

Clinically, the disease was an acute inflammation of the conjunctiva, with no evidence of keratitis. The infection responded to therapy with Neosporin Ophthalmic Ointment; however, it appeared to be self-limiting since some patients recovered without treatment.

The purpose of this study was to characterize by various means the isolates of nontypable *S. pneumoniae*-like organisms obtained from conjunctivae in NYS and compare them with other isolates of nontypable *S. pneumoniae*-like organisms subsequently obtained during outbreaks of conjunctivitis among 80 Marine Corps recruits in San Diego, Calif. (September 1980); 294 university students in Ithaca, N.Y. (February through April 1981); and 1,189 university students in Urbana, Ill. (January through May 1981).
MATERIALS AND METHODS

Bacterial isolates. (i) NYS isolates. From January through June 1980, 125 swabs from affected conjunctival cultures were prepared for aerobic bacteria by the Laboratories for Bacteriology. S. pneumoniae-like organisms were identified by cellular morphology, colony appearance, or blood-agar plates, and the bile-solubility test. The Neufeld capsular-swelling test (2, 14) was then performed with polyvalent and type-specific pneumococcal antisera prepared in this Division. In addition, 66 S. pneumoniae-like isolates obtained from conjunctival swabs by various clinical laboratories in NYS were forwarded to us for the Neufeld test. Seventy-seven swabs were cultured separately for viruses by the Division's Laboratories for Virology.

During the 1981 outbreak in Ithaca, 30 isolates of S. pneumoniae-like organisms were kindly provided by A. B. Ley of the Gamett Health Center, Cornell University, Ithaca, N.Y.

(ii) Isolates from outbreaks outside NYS. Isolates of nontypable S. pneumoniae-like organisms were received from two other outbreaks of conjunctivitis: six isolates obtained from affected eyes in San Diego, Calif., in September 1980 were kindly provided by R. R. Facklam of the Clinical Bacteriology Section of the Centers for Disease Control, Atlanta, Ga.; and nine isolates obtained from affected eyes and the corresponding nasopharynges in Urbana, Ill., during January through April 1981 were kindly provided by Albert Engelbert of the Department of Infectious Disease, University of Illinois, Urbana.

(iii) Control strains. Eleven strains of capsulated S. pneumoniae belonging to types 3, 10, 14, 15, 17, 18, 19, 22 (two strains), 33, and 34 and eight strains of alpha-hemolytic streptococci (three strains of Streptococcus mitis, one strain of Streptococcus salivarius, and four strains of Streptococcus sanguis) were used as controls. These organisms were from the lyophilized culture collection maintained by our laboratory and were originally isolated from clinical specimens.

Media. The media were prepared from either basic ingredients or dehydrated bases by the Division's Media Section.

Biological testing. Ten isolates of nontypable S. pneumoniae-like organisms obtained in various geographic locations were tested by the following biochemical tests: arabinose, dulcitol, glucose, inulin, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sucrose, and xylose fermentations; arginine dihydrolase; 20% bile esculin; bile solubility (tube and plate); catalase; gelatin; indole; 6.5% NaCl; nitrate; optochin disc susceptibility; and Voges-Proskau.

Antibiotic susceptibility testing. Four NYS isolates of nontypable S. pneumoniae-like organisms were tested for antibiotic susceptibilities by the Bauer-Kirby disk diffusion procedure (3). The antibiotic disks used were amikacin, 30 μg; ampicillin, 10 μg; cephalothin, 30 μg; chloramphenicol, 30 μg; clindamycin, 2 μg; erythromycin, 15 μg; gentamicin, 10 μg; kanamycin, 30 μg; methicillin, 5 μg; oxacillin, 1 μg; penicillin, 10 U; streptomycin, 10 μg; tetracycline, 30 μg; and vancomycin, 30 μg. The minimal inhibitory concentration of penicillin was determined by Thornesberry's tube broth-dilution method (18).

Antigen and antibody preparations. Antigen extracts were prepared from all isolates by Lancefield's hot-acid extraction (HAE) procedure (12). Broth-culture antigens were prepared by growing the organisms for 18 to 24 h in Todd-Hewitt broth (THB) and by using the growth medium directly. Purified pneumococcal polysaccharides from capsular types 1 through 40 were prepared in this Division (7) were also used.

Antisera were produced in rabbits against four NYS isolates of nontypable S. pneumoniae-like organisms by using the standard procedure for preparation of Neufeld capsular-swelling antisera (11). Other antisera used included NYS polyvalent and type-specific pneumococcal antisera, Omniserum (Staten Seruminstitut, Copenhagen, Denmark), and antistreptococcal serum groups A, B, C, D, F, and G (purchased from Burroughs-Wellcome Co., Research Triangle Park, N.C.) and groups E, H, K, L, M, and N (produced in rabbits in this Division). Normal rabbit serum was included as a control.

Immunological testing. The antigen and antibody preparations were used in one or more of the following procedures: the Neufeld capsular-swelling test (2, 14), the Lancefield capillary precipitin test (12), and the Ouchterlony immunodiffusion test with double diffusion in 1% Noble agar (15).

Virulence in mice. Female albino Nya:NYLAR mice (18 to 22 g), supplied by the Division's Griffin Laboratory, were inoculated intraperitoneally with various concentrations of four NYS isolates of nontypable S. pneumoniae-like organisms, one strain of typable S. pneumoniae (type 3), and one strain of S. mitis and observed for mortality for 7 days.

Mouse passage. Four NYS isolates of nontypable S. pneumoniae-like organisms were passaged through mice 50 times each. Each time the organisms were grown overnight at 37°C in 5% CO2 in THB. Suspensions adjusted to contain from 106 to 5 × 108 colony-forming units in original growth media were injected intraperitoneally into two to five mice. After 6 h the peritoneal cavity of each mouse was rinsed with THB. The harvested bacteria were plated on blood agar (for study of the organism and to rule out contamination) and inoculated into fresh THB for reincubation overnight and subsequent injection into new mice the next day. The organisms could not practically be passed from mouse to mouse because the number of bacteria recovered was not sufficient to infect new mice.

The organisms recovered from the mice were tested for optochin susceptibility and for the presence of a typable capsule after every five mouse passages.

RESULTS

Epidemiology. The outbreaks of conjunctivitis among college and university students were widespread within NYS during January through June 1980. Of the 1,567 cases reported, 433 (27.6%) occurred in Canton, 410 (26.2%) in Oneonta, 313 (20.0%) in Buffalo, 116 (7.4%) in Albany, 109 (7.0%) in New Paltz, 100 (6.4%) in Troy, and 86 (5.5%) in Ithaca. Monthly reports from these seven institutions indicate that the outbreaks peaked during March and April, with 1,001 cases (63.9%) during these two months.

Culture results. Of the 125 conjunctival swabs...
TABLE 1. Microorganisms isolated from NYS college students with conjunctivitis in 1980

<table>
<thead>
<tr>
<th>Organism(s) identified as:</th>
<th>No. positive</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. pneumoniae,</strong> nontypable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td>30</td>
<td>24.0</td>
</tr>
<tr>
<td>With S. epidermidis</td>
<td>22</td>
<td>17.6</td>
</tr>
<tr>
<td>With S. aureus</td>
<td>2</td>
<td>1.6</td>
</tr>
<tr>
<td>With S. aureus and S. epidermidis</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>With diphtheroids</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>With Acinetobacter calcoaceticus subsp. Iwoffi and Pseudomonas sp.</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Probable pathogens other than pneumococci</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>3</td>
<td>2.4</td>
</tr>
<tr>
<td>H. influenzae, nontypable and diphtheroids</td>
<td>2</td>
<td>1.6</td>
</tr>
<tr>
<td>H. influenzae, nontypable; S. epidermidis and alpha-hemolytic Streptococci</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>H. influenzae, nontypable; S. epidermidis and yeast</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Probable normal flora</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>28</td>
<td>22.4</td>
</tr>
<tr>
<td>Alpha-hemolytic Streptococci</td>
<td>3</td>
<td>2.4</td>
</tr>
<tr>
<td>S. epidermidis and alpha-hemolytic Streptococci</td>
<td>3</td>
<td>2.4</td>
</tr>
<tr>
<td>Diphtheroids</td>
<td>2</td>
<td>1.6</td>
</tr>
<tr>
<td>Others</td>
<td>4</td>
<td>3.2</td>
</tr>
<tr>
<td>No growth</td>
<td>21</td>
<td>16.8</td>
</tr>
</tbody>
</table>

received in our laboratory, 57 (45.6%) contained nontypable *S. pneumoniae*-like organisms (Table 1). No typable pneumococci were isolated. All 66 isolates obtained from conjunctival swabs by other clinical laboratories in NYS and then tested in our laboratories were also nontypable. Ten representative isolates were sent to the Centers for Disease Control and to Statens Serum Institut. Both institutions identified all 10 as nontypable *S. pneumoniae*. In tests of 71 acute and convalescent sera from patients, no serum reacted with HAE antigens from four representative NYS isolates of nontypable *S. pneumoniae*-like organisms in the gel double-diffusion test.

Only 10 of the 125 specimens tested in our laboratory contained other bacterial pathogens. *Staphylococcus aureus* was isolated from six specimens (4.8%), three of which also grew nontypable *S. pneumoniae*-like organisms. Nontypable *Haemophilus influenzae* was isolated from four specimens (3.2%), none containing nontypable *S. pneumoniae*-like organisms.

From the 77 conjunctival swabs received from Canton, Delhi, New Paltz, Syracuse, and Troy and tested by the Division’s Laboratories for Virology, no infectious viral agents were isolated, and 30 paired sera from cases showed no significant rise in titer to adenovirus, influenza A or B virus, measles virus, or herpes simplex virus. Serological tests were also negative for psittacosis-lymphogranuloma venereum and *Mycoplasma pneumoniae*.

Characterization of the *S. pneumoniae*-like isolates. Since the isolates of *S. pneumoniae*-like organisms could not be typed, representative isolates were further characterized. Study was focused on four, one each from Albany, Canton, New Paltz, and Oneonta. Mouse passage was done for possible production of a typable capsule. Each isolate was tested biochemically and immunologically for antibiotic susceptibility and for virulence in mice before and after 50 mouse passages. The isolates were also tested biochemically or immunologically or both ways after 5, 16, 31, and 40 mouse passages. The *S. pneumoniae*-like isolates obtained during the three outbreaks that occurred in September 1980 and January to May 1981 were also nontypable, and representative isolates were studied biochemically and immunologically.

Biochemical testing. The four NYS isolates reacted identically in all 24 biochemical tests: positive for glucose, maltose, and sucrose fer-

FIG. 1. Immunological reactions of HAE antigens from four NYS isolates of nontypable *S. pneumoniae*-like organisms with homologous antisera by immunodiffusion in gel. The antigens, placed in the outer wells, were from isolates obtained in Oneonta (O), Canton (C), New Paltz (NP), and Albany (A). Control antigens were strains of *S. pneumoniae* type 22 (T22) and *S. mitis* (Sm). The antisera, placed in the central wells, were made in rabbits against four representative isolates from Oneonta (Anti O), Canton (Anti C), New Paltz, (Anti NP), and Albany (Anti A).
FIG. 2. Immunological reactions of HAE and THB antigens of nontypable isolates and typable strains of S. pneumoniae with antiserum prepared against the NYS isolate from New Paltz. The antigens, placed in the outer wells, included: (a) NYS isolates from Oneonta (O), Canton (C), New Paltz (NP), and Albany (A) and S. pneumoniae type 3 (T3), and S. mitis (Sm); (b) O, C, A, and other outbreak strains from San Diego (S), Ithaca (I) and Urbana (U); (c), O, C, A, and strains of S. pneumoniae types 3, 10, and 14 (T3, T10, T14). The antiserum was placed in the central wells.

mentation, for bile solubility, and for optochin susceptibility; negative in the other 18 tests.

Six isolates from the other outbreaks, two each from San Diego, Ithaca, and Urbana, were also tested biochemically. Four of the six reacted in the same manner as the 1980 NYS isolates. One isolate from Ithaca and one from Urbana were positive in lactose and raffinose fermentation tests; the same isolate from Ithaca was also positive in the arginine dihydrolase test.

Antibiotic susceptibility testing. The antibiotic susceptibility zone diameters were similar for the four NYS isolates, using the Bauer-Kirby disk diffusion procedure (3). The isolates were resistant to amikacin, kanamycin, and streptomycin and susceptible to all other antibiotics used. The minimal inhibitory concentration of penicillin was 0.16 U/ml for all four isolates.

Immunological reactions. The NYS isolates were not positive for capsular swelling with NYS pneumococcal antisera, Omniserum, or autologous antisera. Even though each isolate was passed through mice 50 times, no typable capsule resulted.

No reaction was seen between the HAE antigens of the four NYS isolates and antistreptococcal sera of groups A through H and K through N in the capillary precipitin test.

The HAE and THB antigens from all four NYS isolates reacted strongly in the gel double-diffusion test with all four homologous rabbit antisera. With both antigen preparations, lines of identity were observed between each isolate and all four antisera (Fig. 1 and 2).
HAE antigens from 9 of the 11 strains of typable *S. pneumoniae* tested were nonreactive with the antisera to the four NYS isolates in the gel double-diffusion test. HAE antigen from the other two strains, types 3 and 22, showed some reaction with antiserum to the New Paltz isolate, but the precipitin line was not identical to those formed with the four NYS isolates (Fig. 1 and 2). In contrast, THB antigens from the 11 typable strains did react with all four NYS antisera, each showing a line of identity with the other, and when 3 of these strains (types 3, 10, and 14) were tested together with 3 of the NYS isolates, a line of identity was seen among antigens from the typable strains and nontypable isolates (Fig. 2c). However, a faint outer precipitin line seen with the NYS isolates was not shared by the typable strains. Neither antigen preparation of the *S. mitis*, *S. salivarius*, and *S. sangiu* isolates showed any reaction in this test (Fig. 2a).

HAE and THB antigen preparations from isolates obtained during the three more recent outbreaks reacted with the four antisera in the gel double-diffusion test as follows. The six isolates from San Diego, the three from Ithaca, and seven of the nine from Urbana reacted with all four antisera and showed lines of identity with each other and with the antigen preparations from the four NYS isolates. The other two isolates from Urbana showed no reaction. These two immunologically nonreactive isolates were recovered from the eye and nasopharynx of the same student. One isolate was also tested biochemically and differed from the other isolates.

Purified pneumococcal polysaccharides from capsular types 1 through 40 reacted with the four antisera as follows: 34 types reacted with the New Paltz isolate antiserum; 28 reacted with the Oneonta isolate antiserum; 27 reacted with the Canton isolate antiserum; and 19 reacted with the Albany isolate antiserum. Five capsular types (types 2, 3, 21, 27, and 31) did not react with any of the four antisera.

Virulence in mice. All four NYS isolates were relatively nonpathogenic in mice since intraperitoneal injection of 10<sup>10</sup> of each did not kill the mice. When the control strains were injected intraperitoneally the mice were killed by 10<sup>10</sup> *S. mitis* and by 10<sup>5</sup> *S. pneumoniae* type 3.

Gradual changes in the four NYS isolates after mouse passage. Although the original reason for mouse passage was to determine capsular production, some other characteristics of the organisms proved to be of interest. After five mouse passages, bile solubility, optochin susceptibility, and reactivity in autologous antiserum in the gel double-diffusion test were still seen. After 16 passages, bile solubility and optochin susceptibility were permanently lost by all four isolates. After 31 passages, three of the isolates became lactose-positive. After 40 passages, a partial loss of reactivity in autologous antisera was seen (HAE antigens from the isolate from Oneonta still reacted with all four antisera, but the other isolates reacted only with the antiserum from New Paltz). After 50 passages, all four isolates were bile- and optochin-negative, lactose-positive, and reactive only with the antiserum from New Paltz.

The antibiotic susceptibility tests were done only with the original isolates and with the isolates after 50 mouse passages. Susceptibility to all antibiotics tested either did not change or changed in a similar manner for all four isolates. After 50 passages, the isolates demonstrated a slightly increased resistance to the penicillins, cephalothin, and tetracycline and a decreased resistance to amikacin (Fig. 3). The minimal inhibitory concentration of penicillin exhibited the same trend: an increase from 0.16 to 0.25 U/ml with all four isolates.

After 50 mouse passages, the isolates were more virulent in mice since an injection of 3 × 10<sup>8</sup> to 4 × 10<sup>8</sup> organisms of any isolate was fatal.
DISCUSSION

The nationwide outbreaks of conjunctivitis have focused attention on nontypable *S. pneumoniae*-like organisms isolated from eyes. The virulence mechanism of this variant remains in question because the known pathogenic factor of pneumococci is the antiphagocytic role of the polysaccharide capsule. Several observations suggest that the fate of a polysaccharide capsule in eyes is uncertain. Almost one-quarter of the pneumococcal conjunctival isolates from a 39-year study were nontypable (8), whereas in the same study <1% of the respiratory tract isolates were nontypable. In the recent outbreaks of conjunctivitis all of the *S. pneumoniae*-like isolates were nontypable. Similarly, all *H. influenzae* isolated from conjunctivae in our study and in a recent study in children (10) were nontypable. It is unclear whether tear lysozyme, selection for nonencapsulated variants by specific immunoglobulin A or unknown mechanisms in eyes could contribute to the loss of capsular material. In the four 1980 NYS isolates we studied, the capsular loss was irreversible; no typable capsule occurred, even after 50 passages through mice. However, other cell surface changes such as partial loss of reactivity in autologous antisera, loss of autolysis in the presence of bile, and increased resistance to penicillin were seen.

A close relationship was established among the four isolates from distinct geographic locations within NYS in 1980, based on the following observations: all four original isolates reacted in the same manner biochemically, immunologically, and in antibiotic susceptibility and mouse virulence tests; and all characteristics tested either did not change after 50 mouse passages or gradually changed in a similar manner, although not at the exact same mouse passage for all four isolates. In addition, no plasmid bands were seen in any of the four isolates on agarose gel electrophoresis (13).

Austrian, while studying morphological variants of pneumococci, has found that both capsulated and noncapsulated strains possess a species-specific C-carbohydrate antigen (1). Freeman states that pneumococci also share a common somatic antigen (9). In the present study the gel double-diffusion test with antisera to the four NYS isolates demonstrated an antigen common to all types of capsulated *S. pneumoniae* tested and to the nontypable isolates, but not to eight isolates of *S. mitis*, *S. salivarius*, and *S. sanguis*. With counterimmunoelectrophoresis and Omniserum, the common pneumococcal antigen was also demonstrated as a faint precipitin line between the four NYS isolates and one strain of typable pneumococci, but not *S. mitis*: A strong precipitin line, most probably due to the presence of a specific capsular type, was seen in this test only with the typable strain. Further testing revealed that although the common pneumococcal antigen was present on the four NYS isolates, type-specific capsular antigens could not be demonstrated either by our Neufeld capsular-swelling tests with NYS antisera or by counterimmunoelectrophoresis with Danish type-specific antisera.

Overall, the nontypable isolates proved to be antigenically more similar to each other than to the typable capsulated strains. No antigenic similarity was observed between the nontypable isolates and the eight isolates of alpha-hemolytic streptococci. Hot-acid-extracted antigens from the 4 NYS isolates and from 16 of the 18 isolates obtained during three other nationwide outbreaks reacted strongly with the 4 NYS antisera and showed line(s) of identity with each other. This more specific antigenic relationship not shared by the typable capsulated pneumococci suggests that the nationwide outbreaks of 1980 and 1981 were caused by closely related organisms, an inference that has been elusive in the absence of a typable capsule.

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LITERATURE CITED


