BOX–Polymerase Chain Reaction–Based DNA Analysis of Nonserotypeable Streptococcus pneumoniae Implicated in Outbreaks of Conjunctivitis

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Nonserotypeable isolates predominate in epidemic conjunctivitis caused by Streptococcus pneumoniae. Previous evaluations of outbreaks of pneumococcal conjunctivitis have relied on epidemiologic factors and the nontypeability of the isolates to infer that a single clone was involved. In the present study, BOX–polymerase chain reaction DNA analysis was used to characterize nonserotypeable S. pneumoniae isolated by conjunctival culture during a recent conjunctivitis outbreak and to compare these isolates with those from outbreaks described earlier. The recent outbreak was caused by a single pneumococcal clone. Outbreaks in separate parts of the United States in 1980–1981 were all caused by the same clone. Cluster analysis revealed a high degree of genetic relatedness among isolates causing conjunctivitis compared with that among other nonserotypeable S. pneumoniae, with the closest relatedness being found among the 1996 and 1980–1981 conjunctival isolates.

Ample data obtained in vitro and in vivo in experimental animals [1] demonstrate that unencapsulated isolates of Streptococcus pneumoniae are far less virulent than encapsulated ones. Although only rarely implicated as a cause of invasive human disease [2], these nonserotypeable organisms appear to have a predilection for infecting the conjunctivae and, especially, for causing epidemic conjunctivitis [3, 4]. A careful analysis by Shayegani et al. [3] showed that among 123 conjunctival isolates of S. pneumoniae, many of which had been obtained during outbreaks of infection, none were serotypeable.

Whereas epidemiologic study of outbreaks due to encapsulated pneumococci can be accomplished (at least in part) by the use of serotyping, this method is not helpful in the case of nonserotypeable organisms. In recent years, molecular biologic techniques have been used to further characterize or subtype S. pneumoniae. BOX repeat element–based polymerase chain reaction (BOX-PCR) is a rapid and highly reproducible method for quantitating genotypic variation among pneumococcal isolates [5, 6] that does not rely on phenotypic characters; its capacity to discriminate between similar but not identical isolates meets or exceeds that of other available molecular typing approaches [5, 6]. This technique has been successfully used in the evaluation of both serotypeable [5, 6] and nonserotypeable pneumococci [7].

During a recent outbreak of conjunctivitis at a naval training station, bacterial cultures revealed nonserotypeable S. pneumoniae as the predominant organism causing infection. We used BOXA1R-based PCR to characterize isolates from this outbreak as well as from outbreaks described earlier by Shayegani et al. [3]. To our knowledge, this is the first report describing genetic relatedness among nonserotypeable pneumococcal isolates.

Methods

Outbreak. Between 29 January and 26 April 1996, 561 cases of conjunctivitis were seen in an outpatient clinic that provides care for students at a navy training center in Great Lakes, Illinois. A case was defined as any clinical diagnosis of conjunctivitis in a trainee who had not been diagnosed with conjunctivitis in the previous 14 days. Once the outbreak was recognized, the Preventive Medicine Division requested that samples of the conjunctival sac be cultured; however, compliance with this request was limited by health care provider awareness and by the availability of culture swabs. Cultures were, therefore, obtained from 97 cases seen in the middle to late course of the outbreak. Samples for bacterial culture, obtained using a cotton-tipped swab, were delivered to the laboratory in transport medium (Starplex Scientific, Etobicoke, Canada) within 2 h and were streaked onto blood, chocolate, and McConkey agar. For 33 patients, specimens were also obtained using a rayon-tipped swab delivered in transport medium (Difco,
Detroit) as above and cultured to evaluate for the presence of adenoviruses, enteroviruses, and herperviruses.

Identification. Isolates were identified as *S. pneumoniae* on the basis of the following: α-hemolytic activity during growth on tryptic soy agar (TSA) containing 5% sheep blood, morphology, susceptibility to optochin, and solubility in 1% sodium deoxycholate. Identification was further confirmed using a culture identification test (AccuProbe; GenProbe, San Diego), which detects RNA sequences unique to *S. pneumoniae*, and by reactivity with monoclonal antibody HASP to pneumococcal wall polysaccharide (Statens Seruminstitut, Copenhagen). Organisms identified as pneumococci were stored without further passage on TSA-blood agar plates at 4°C and frozen in whole defibrinated sheep blood at −70°C.

Serotyping. Pneumococci were cultured overnight on TSA containing 5% sheep blood. Colonies were harvested and suspended in PBS with 2% formalin to yield 5×10⁶ cfu/mL. Serotyping was attempted by coagglutination after the method of Kronvall [8] using the checkerboard procedure of Sørensen [9], which uses two sets of pooled antisera (Statens Seruminstitut). Agglutination of typeable pneumococci is detected visually without magnification within 1 min. When isolates did not appear typeable by this method, Omniserum (Statens Seruminstitut), which contains antibodies to 83 pneumococcal polysaccharide serotypes, was used to exclude the possibility that a polysaccharide capsule of an unusual serotype was present.

For each study, negative control samples containing no antiserum and positive controls containing encapsulated pneumococci with antiserum directed against the homologous pneumococcal polysaccharide serotypes were included. All samples were prepared by 1 technician, who then coded the slides prior to reading by a single investigator. All typing studies were done at least twice.

**BOX-PCR.** After growing overnight on TSA containing 5% sheep blood, bacterial colonies were harvested, washed once in 1 M NaCl, washed twice in distilled water, and resuspended to 10⁷–10⁸ cfu/mL in distilled water. Using the BOXA1 Roligonucleotide primer [5], PCR was performed using whole cell suspensions of pneumococci to provide template DNA [7]. Each 25-μL PCR reaction mixture contained 50 μM primer, 1.25 mM each of the four dNTPs (Promega, Madison, WI), 2 U of Taq DNA polymerase (Perkin-Elmer, Forest City, CA) in a buffer containing 10% DMSO, and 2 μL of the bacterial suspension, all added to high-performance liquid chromatography–grade water. PCR amplifications were done in an automated thermal cycler (Perkin-Elmer). The initial mixture was heated to 80°C for 15 min, after which the primer and Taq DNA polymerase were added. This step was followed by 30 cycles, each consisting of denaturation at 95°C for 30 s, annealing at 52°C for 1 min, and extension at 65°C for 8 min, followed by a final extension at 65°C for 15 min. Seven microliters of each PCR product was subjected to electrophoresis in a 1% agarose gel at 100 V for 2.5 h in standard TRIS borate–EDTA electrophoresis buffer and stained with 0.5 μL/mL ethidium bromide. Agarose gels were visualized and photographed under UV transillumination, and DNA banding patterns were analyzed using the RFLPscan software package (Scananalytics, Billerica, MA) according to the instructions of the manufacturer. The DNA banding patterns obtained from each isolate were loaded into a database and analyzed using the bin method, which matches bands that have like molecular weight values. Dendrograms were generated using TreeCon (Scanalytics) with data derived from the RFLPscan database.

**Results**

**Clinical characteristics of cases.** Clinical presentation of conjunctivitis cases was relatively benign; no concurrent pneumonia, sinusitis, or otitis media was noted in association with conjunctivitis. Approximately 40% of cases presented with, or developed, bilateral eye involvement. Purulence was occasionally noted but was not a hallmark of this outbreak. All cases were treated with topical antibiotics (usually erythromycin ointment). The average duration of symptoms was <7 days. Epidemiologic analysis revealed no common source, location, or contacts associated with the conjunctivitis cases; all environmental cultures (*n* = 11) were negative. When cases were compared with the overall navy training population (those who enlisted between August and December 1995), no statistically significant differences were found in age, gender, race, smoking, use of corrective lenses, or history of eye disease.

**Outbreak isolates.** Cultures for a bacterial etiology were obtained in 97 cases of conjunctivitis. *S. pneumoniae* was isolated from 32 (33%) of these specimens, *Haemophilus influenzae* from 2, and mixed bacterial flora (speciation not attempted) from 8; the remaining 55 specimens showed no growth. Viral cultures were done for 33 cases, and all were negative. Eight pneumococcal isolates (representing late-outbreak cases) were available for further study and will be referred to in the present paper as 1996 outbreak isolates.

**Other pneumococcal isolates.** In addition to the 1996 outbreak isolates, we studied 22 others (table 1), all of which met our criteria for nonserotypeable *S. pneumoniae*. These included: 2 isolates from Fargo, North Dakota, from patients with conjunctivitis (hereafter called 1995 conjunctival isolates); 14 isolates from 6 outbreaks of pneumococcal conjunctivitis (2 or 3 from each outbreak) that occurred in 1980–1981 [3]; and 6 isolates derived from other body sites (5 sputum, 1

<table>
<thead>
<tr>
<th>Geographic origin</th>
<th>Year</th>
<th>Clinical source</th>
<th>No. of isolates*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great Lakes, IL</td>
<td>1996</td>
<td>Conjunctival</td>
<td>8 (1–8)</td>
</tr>
<tr>
<td>Houston</td>
<td>1993–1995</td>
<td>Nonconjunctival¹</td>
<td>6 (9–14)</td>
</tr>
<tr>
<td>Fargo, ND</td>
<td>1995</td>
<td>Conjunctival</td>
<td>2 (15, 16)</td>
</tr>
<tr>
<td>Chicago</td>
<td>1981</td>
<td>Conjunctival</td>
<td>3 (17–19)</td>
</tr>
<tr>
<td>Itaca, NY</td>
<td>1980</td>
<td>Conjunctival</td>
<td>2 (20, 21)</td>
</tr>
<tr>
<td>New Paltz, NY</td>
<td>1981</td>
<td>Conjunctival</td>
<td>2 (22, 23)</td>
</tr>
<tr>
<td>Oneonta, NY</td>
<td>1980</td>
<td>Conjunctival</td>
<td>2 (24, 25)</td>
</tr>
<tr>
<td>San Diego</td>
<td>1981</td>
<td>Conjunctival</td>
<td>3 (26–28)</td>
</tr>
<tr>
<td>St. Lawrence, NY</td>
<td>1980</td>
<td>Conjunctival</td>
<td>2 (29, 30)</td>
</tr>
</tbody>
</table>

* Nos. in parentheses correspond to isolate no., as shown in figures 1 and 2. ¹ Five from sputum, 1 from bronchoalveolar lavage specimen.

**Table 1.** Characteristics of nonserotypeable *S. pneumoniae* isolates evaluated by BOX-polymerase chain reaction–based DNA analysis.

bronchoalveolar lavage) cultured from subjects seen at the Houston VA Medical Center during the past 4 years.

**Serotyping.** Resuspension of nonserotypeable pneumococci to the desired concentration in PBS with formalin was followed, within a few minutes, by the appearance of autoagglutination. This reaction developed more slowly than that caused by admixing typeable pneumococci and type-specific antiserum. Compared with positive controls (encapsulated organisms), outbreak isolates did not react in pooled antisera or Omniserum. Concordant results were obtained independently by two participating laboratories using coded specimens.

**BOX-PCR.** The 8 outbreak isolates from 1996 displayed identical banding patterns by BOX-PCR (figure 1A). The 2 1995 conjunctival isolates, except for one minor band, shared a common DNA pattern. Of note, isolates obtained in 1980–1981 from outbreaks in New York State, Illinois, and California were identical (figure 1B). Six nonserotypeable pneumococci from diverse sources showed a variety of banding patterns (figure 1A). The patterns observed in nonserotypeable *S. pneumoniae* were distinct from the prevalent genotypes observed in commonly isolated serotypeable organisms (data not shown). A dendrogram (figure 2), constructed using cluster analysis,
showed that all conjunctival isolates were more closely related to each other than to other nonserotypeable pneumococci, with the closest relatedness (>76%) being found among the 1996 and 1980–1981 isolates.

Discussion

During studies of conjunctivitis outbreaks in young adults, Shayegani et al. [3] isolated S. pneumoniae from 46% of all conjunctival samples and from 89% of samples in which pathogenic bacteria were identified; all of these pneumococci were unencapsulated. Viral cultures were uniformly negative. These results, obtained nearly 2 decades ago, are strikingly similar to those from the present outbreak. They suggest that nonserotypeable pneumococci may exhibit some as yet poorly understood tropism for conjunctival tissue, although it is worth noting that sporadic conjunctivitis is more likely to be due to organisms other than pneumococcus [10–12], and the majority of pneumococcal isolates recovered from patients with sporadic conjunctivitis are typeable [13].

The present study utilized molecular biologic techniques unavailable during the 1980–1981 outbreak to add to our understanding of the role of unencapsulated pneumococci in conjunctivitis outbreaks. While Pease et al. [4] were able to identify atypical α-hemolytic streptococci causing conjunctivitis as unencapsulated pneumococci on the basis of SDS-PAGE profiles, our results support their identification as S. pneumoniae by showing that all the isolates analyzed contain RNA sequences and cell-wall polysaccharide unique to S. pneumoniae. The use of BOX-PCR–based DNA analysis conclusively demonstrated that the 8 pneumococcal isolates isolated from infected subjects during the 1996 outbreak comprised a single clone. Earlier studies of outbreaks caused by unencapsulated pneumococci used biochemical testing, immunologic reactions, and/or antibiotic susceptibility profiles [3] to demonstrate that each outbreak was the result of the spread of a single clone.

By using BOX-PCR, we were able to show that the 1996 outbreak isolates were more closely related to isolates from persons who had conjunctivitis in North Dakota in 1995 than to other nonserotypeable pneumococci. More striking, however, was the finding that nonserotypeable pneumococci causing epidemic conjunctivitis in widely separated areas of the United States during 1980–1981 shared identical DNA banding patterns. Although these isolates were different from those isolated in 1995 or 1996, there was a remarkable degree of relatedness among all conjunctivitis isolates compared with that of other nonserotypeable pneumococci.

It is not certain why unencapsulated pneumococci have a propensity to cause conjunctivitis. The capacity of pneumococci to cause inflammation is largely due to the cell wall, not the capsule, although both may participate [14, 15]; accordingly, inflammation is an expected outcome if conditions allow bacterial proliferation. Although unencapsulated pneumococci are expected to be readily opsonized by serum constituents, such as the ubiquitous antibody to the pneumococcal cell-wall polysaccharide and complement, and hence to be infrequently implicated as a cause of invasive disease, the absence of antibody and complement in the conjunctival sac might provide an environment that permits the development of infection. These observations do not, however, explain the relative infrequency with which encapsulated pneumococci have been implicated in epidemic conjunctivitis. Suppression or deletion of capacity to produce an extracellular capsule may facilitate the interactions of cell-wall polysaccharide and one or more surface expressed pneumococcal proteins with the conjunctival surface. These or other bacterial factors responsible for the capacity of closely related nonserotypeable isolates to cause conjunctivitis remain to be elucidated.

References

Human granulocytic ehrlichiosis (HGE) is a tickborne infection first described in 1994 [1]. This disease has tentatively been given the name of HGE because colonies of intracellular bacteria (morulae) are often seen within peripheral blood granulocytes. This feature helps to clinically distinguish HGE from ehrlichiosis caused by *Ehrlichia chaffeensis*, in which infection of peripheral blood monocytes is observed [2]. HGE is caused by an obligate intracellular gram-negative bacterium, which is genetically closely related, if not identical, to the ehrlichiae that infect horses and ruminants, *Ehrlichia equi* and *Ehrlichia phagocytophila*, both of which also infect peripheral blood granulocytes [3–5].

The clinical manifestations of HGE include fever, myalgias, and headache. Infection may be severe, with renal, pulmonary, and neurologic complications, and deaths have been reported in up to 5% of cases [6]. Most patients have elevated hepatic transaminase levels and significant hematologic abnormalities, including thrombocytopenia and leukopenia [1, 6].

The spectrum of host cells and tissues infected with the HGE agent remains unknown. So far, mature granulocytes are the

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Informed consent was obtained from all patients before samples were obtained, in accordance with guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota.

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