Analysis of the Genetic Structure of Nontypeable Pneumococcal Strains Isolated from Conjunctiva

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Received 23 April 2004/Returned for modification 17 August 2004/Accepted 7 October 2004

More than 50% of the nontypeable (NT) pneumococcal strains received in our laboratory for reference purposes are isolated in sporadic cases of conjunctivitis. To determine the genetic structure of the population of these NT conjunctival strains, we analyzed 75 pneumococci (40 NT and 35 typeable) isolated from conjunctivitas and 30 (15 NT and 15 typeable) isolated from other sources. The NT and typeable conjunctival strains grouped in separate clusters, whereas NT and typeable pneumococci isolated from other sources were similarly distributed. NT conjunctival strains belonged to two well-differentiated clonal lineages. The first, represented by three newly described sequence types, featured fully antibiotic susceptible strains and appeared to be characteristic of conjunctival tissue; the second, represented by the previously described ST344, had a pattern of multiresistance to penicillin, tetracycline, and erythromycin and shared a genetic background with some NT strains isolated from other sources.

Streptococcus pneumoniae is an important pathogenic bacterium associated with pneumonia, septicaemia, meningitis, and otitis. It is also a common cause of acute conjunctivitis, particularly in children, but also in adults (11, 19). For reference purposes, our laboratory receives many pneumococcus samples from all origins that have been isolated in Spanish hospitals (8). Around one-third of the strains isolated from children below the age of 6 months that were studied in our laboratory between 1990 and 1999 caused acute conjunctivitis (9).

Pneumococcal serotyping usually fails to detect a small number of strains that do not react with antipneumococcal typing sera. Nontypeable (NT) strains are infrequently isolated from sterile clinical specimens (2.2%), in which case they are rarely implicated as causes of invasive disease (2, 12), since they are otherwise relatively common in nonsterile samples (10%). The identification of these NT pneumococci is dubious (14, 17), particularly in nonsterile specimens, and they may be confused with other Streptococcus species. The association between the presence of NT isolates and the occurrence of conjunctivitis was first suggested in 1977 (10) in a retrospective study of the incidence of capsular types in a Boston hospital between 1935 and 1974.

Further studies associated NT strains with outbreak and sporadic cases of conjunctivitis (1, 6, 18, 20), and a recent report has confirmed NT S. pneumoniae-like strains isolated from an outbreak of epidemic conjunctivitis as being S. pneumoniae (3).

In the last 10 years, approximately 50% of the NT pneumococcal strains received in our laboratory have been isolated from cases of conjunctivitis, and the frequency of these noncapsular strains was five times that found in other pathologies (laboratory data).

In general, the NT strains have been characterized in cases related to outbreaks, but as yet we have little information concerning those strains isolated from sporadic cases.

The purpose of this study was to characterize NT pneumococcal strains isolated from conjunctivitas in sporadic cases of conjunctivitis in Spain between 1997 and 2002. The overall objective was to determine whether the population genetic structure of this group of strains was similar to that found in typeable strains isolated from conjunctivitas. In addition, the genetic relatedness of NT isolates from conjunctivitas and NT strains from other origins was also analyzed. Pulsed-field gel electrophoresis (PFGE) (15) and multilocus sequence typing (MLST) (5) molecular markers were used for these pneumococcal strains.

MATERIALS AND METHODS

Strains. (i) Identification and typing. A total of 14,650 pneumococcal isolates were received in our laboratory between 1997 and 2002. Of these, 1,068 strains were isolated from conjunctivitas.

All isolates were identified as S. pneumoniae by their distinctive colony morphology on sheep blood agar and were confirmed by the optochin susceptibility and bile solubility tests. Serotyping was initially performed with a dot blot assay as previously described (7), using 46 anticapsular sera provided by the Statens Serum Institut (Copenhagen, Denmark). Strains that gave uncertain results with this technique were typed by the Quellung reaction. Strains that did not react at all with any sera were classified as NT.

All NT strains were confirmed as S. pneumoniae by using the AccuProbe S. pneumoniae identification kit (Gene Probe, Inc., San Diego, Calif.) (4).

(ii) Strains analyzed by PFGE. Thirty-five NT and 25 typeable strains isolated from conjunctivitas of different geographical origins within Spain between 1997 and 2000 were chosen at random to be analyzed by PFGE.

(iii) Strains analyzed by MLST. Sixty pneumococcal strains were analyzed by MLST. Fifteen of these (5 NT and 10 typeable strains) represented the most frequent profiles obtained by PFGE analysis. Another 15 (10 NT and 5 typeable strains) were selected from the conjunctival strains isolated in 2001 and 2002. Finally, 30 strains (15 NT and 15 typeable) were selected from isolates recovered from other sources during the same period.

PFGE. Genomic DNA was prepared in agarose blocks as previously described (13) and digested with Smal (MBI Fermentans, Quimigranel, Spain) in the recommended restriction buffer. PFGE was performed in 1% agarose MP (Roche Diagnostics Corporation, Indianapolis, Ind.) in 0.5× Tris-borate-EDTA buffer at 12°C and 6 V/cm in a CHEF-DR II (Bio-Rad Laboratories, Hercules, Calif.) for 22 h with a switching time of 0.1 to 40 s.

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Band patterns were compared by using the Molecular Analyst Fingerprint (Bio-Rad Laboratories). A dendrogram was generated from the data by the unweighted-pair group method using average linkages, with the Dice coefficient and a tolerance of 1% (see Fig. 2).

MLST. Briefly, internal fragments of the aroE, gdh, gki, recP, spi, xpt, and ddl genes were amplified and then sequenced in each direction with primers described by Enright and Spratt (5). The sequences of each of the seven loci were compared with those of all the known alleles, by using the programs available on the MLST website (http://www.mlst.net/) to assign allele numbers. The same software was used to define the allelic profile and sequence type (ST) of each isolate. Data were analyzed, and the appropriate dendrogram was generated (see Fig. 3), with a sequence type analysis and recombinational test.

RESULTS

The conjunctival strains represented 7.2% of all pneumococcal isolates studied between 1997 and 2002. This percentage is similar to that found for otic strains (6.9%), while invasive isolates (i.e., isolates from blood or cerebrospinal fluid) recovered in the same period represented 45%.

NT strains were the most frequent (23.2%) of the conjunctival isolates. This NT percentage is higher than those found for strains isolated from other sources (Fig. 1). The most representative serotypes of the pneumococci causing conjunctivitis in Spain during the period 1997 to 2002 were 19A/F (14.1%), 6B (9.8%), 23F (8.8%), 14 (5.3%), 6A (4.6%), 23A/D (3.7%), and 11 (3.3%). This distribution does not differ significantly from that of serotypes found among pneumococci isolated from cases of invasive disease.

PFGE analysis. Digestion of DNA with SmaI yielded a total of 39 different band pattern profiles. Nineteen of the 35 (54.3%) NT strains from conjunctivas corresponded to only four closely related profiles, while the 25 typeable strains corresponded to 22 profiles (Fig. 2).

In general, the isolates were distributed in clusters in accordance with their typeability. NT isolates appeared in two clusters, A and C (Fig. 2), and the typeable pneumococci were grouped in cluster B.

MLST typing analysis. The relationships based on the allelic profiles of the 60 pneumococcal isolates analyzed by MLST are shown in Fig. 3. Thirty-three STs were found. Nevertheless, all of the NT strains isolated from conjunctivas belonged to only four STs, three of which (ST941, ST942, and ST943) were closely related. The pneumococci grouped in these three related STs belonged to PFGE cluster A (Fig. 2), and their allelic profiles shared six of the seven alleles analyzed. However, these strains shared only two or three of their alleles with the fourth profile (ST344) (Fig. 3).

The NT pneumococcal strains isolated from other sources were mainly distributed in other STs (Fig. 3) in the dendrogram. It was particularly striking that the three related STs corresponded solely to NT strains isolated from conjunctivas, while ST344 grouped not only NT pneumococci isolated from conjunctivas but also other NT strains isolated from different sources. Likewise, typeable strains isolated from conjunctivas had no particular distribution pattern, appearing in different STs with typeable strains isolated from other sources.

DISCUSSION

Sporadic conjunctivitis has traditionally been associated with different microorganisms (11), including typeable pneumococcal strains (10). However, NT pneumococcal strains have been proposed to be an important cause of infection, particularly in outbreaks of conjunctivitis (18, 20). Nontypeability may result from a loss of capsular material due to specific but poorly understood mechanisms (20). In fact, to express little or no capsular polysaccharide can be advantageous in that it allows the colonization of several tissues, including the conjunctival surface (1, 21).

In this study, a population of NT pneumococcal strains isolated from conjunctivas in sporadic cases was analyzed by comparing it with other populations of strains (typeable and NT) isolated from conjunctivas and other sources. Between 20.5 and 28.7% of the conjunctival pneumococci studied in our laboratory were NT (Fig. 1), a frequency similar to that described by other authors (10, 20). This contrasts with the low percentage (2.2%) of NT strains isolated from other sources.
(2) and with the infrequent appearance of NT strains in invasive diseases (12).

According to the data obtained by PFGE (Fig. 2), NT conjunctival strains were distributed in two separate clusters, A and C, which were well differentiated from that of the typeable isolates of the same origin. This suggests that NT strains isolated from conjunctivas may possess particular genetic characteristics suggesting a clonal nature.

On the basis of MLST, NT strains from conjunctivas again formed a group separate not only from the typeable strains isolated from conjunctivas but also from typeable strains of other origins, thereby yielding two distinct and well-differentiated clonal lineages, one made up of STs 941, 942, and 943, and one consisting of ST344 (Fig. 3). However, most of the patterns found in NT strains isolated from other sources were grouped with typeable isolates and had common or closely related STs. These may be considered pneumococci with similar genetic backgrounds that express little or no capsular polysaccharide.

The population structure of the typeable pneumococci isolated from eyes is similar to that found in all pneumococcal strains (13). Throughout the dendrogram, typeable strains isolated from eyes and from other sources were grouped within the same clonal lineages. Some typeable pneumococcal strains are thus able to survive in conjunctivas and give rise to sporadic cases of conjunctivitis. NT strains of STs 941, 942, 943, and 344 might be better adapted to the colonization of eye tissue. However, nontypeability alone cannot explain the specific location of these pneumococcal strains in the eyes, since most of the NT pneumococci isolated from other origins did not belong to these two clonal lineages. The isolates belonging to ST344 (Fig. 3) showed a typical pattern of multiresistance to penicillin, tetracycline, and erythromycin, while the other NT isolates (ST941, -942, and -943), grouped in the other cluster, were all susceptible. ST344 falls within cluster C by PFGE. Two strains with this ST, isolated from blood, have been described in the MLST database previously: one had the multiresistant pattern, and the other was resistant to penicillin but not to erythromycin. Therefore, NT ST344 pneumococci might not represent a clonal lineage exclusive to the conjunctiva. In fact, in our study, some NT strains from other sources were found in this ST. To address this matter, a large number of strains of this specific ST need to be analyzed.

By contrast, the other NT pneumococci isolated from the
FIG. 3. Dendrogram derived from cluster analysis of allelic profiles generated by MLST analysis of typeable and nontypeable pneumococcal strains isolated from conjunctivas (marked with asterisks) and other sources.
eye, which grouped together in three closely related STs, corresponded to cluster A and might represent a characteristic conjunctival-tissue cluster. However, other authors have not found specific clusters among NT strains isolated from sporadic cases of conjunctivitis (1). In the previous study, BOX-PCR was used to characterize the isolates; this methodology might not reflect the genetic structure of the pneumococcal population analyzed as well as MLST does.

The NT strains belonging to the two clonal lineages described in this study might represent Spanish endemic pneumococcal clones involved in conjunctivitis cases over a long period. In fact, several other NT pneumococci isolated from conjunctivacys in Spain many years before, in the 1980s, also belong to the same clonal lineages (laboratory data). Two previous studies have shown that closely related NT strains were responsible for conjunctivitis outbreaks in different geographical areas over at least 20 years (6, 16).

Further studies including a larger number of NT pneumococcal strains isolated from conjunctivacys and other tissues from different and widely separated countries are required to confirm our findings. Otherwise, we cannot discount the possibility that these NT pneumococci represent a group genetically divergent from typical pneumococcal strains. Additional studies with these strains, including the analysis of the capsular operon but also some other genes, might better clarify their real phylogenetic position.

ACKNOWLEDGMENTS

This work was supported by The Spanish Pneumococcal Infection Study Network, Red Temática de Investigación Cooperativa (G03/103), Ministerio de Sanidad.

We thank the laboratories that sent us the pneumococcal strains.

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