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Invasiveness of Serotypes and Clones of *Streptococcus pneumoniae* among Children in Finland

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Streptococcus pneumoniae **(the pneumococcus) causes diseases from otitis media to life-threatening invasive infection. The species is extremely antigenically and clonally diverse. We wished to determine odds ratios (ORs) for serotypes and clones of *S. pneumoniae* that cause invasive disease in Finland. A total of 224 isolates of *S. pneumoniae* from cases of invasive disease in children <2 years of age in Finland between 1995 and 1999 were serotyped, and sequence types (STs) were determined by multilocus sequence typing. These STs were compared with a previously published carriage data set. STs from invasive disease were significantly less diverse than those from carriage (invasive disease, 0.038 ± 0.01; carriage, 0.019 ± 0.005). The ORs of serotypes 14, 18C, 19A, and 6B were significantly greater than 1, indicating association with invasive disease. The ORs of 6A and 11A were significantly less than 1. The difference between 6A and 6B is significant, which suggests that relatively subtle changes in the capsule may have a dramatic effect upon disease potential. We found that ST 156, the Spain^{9V}-3 clone which mainly expressed serotype 14 in Finland, is strongly associated with invasive disease (OR, 10.1; 95% confidence interval, 1.3 to 79.5). Significant associations with invasive disease were also detected for STs 482, 191, 124, and 138, and associations with carriage were detected for STs 485 and 62. These results demonstrate the invasive phenotype of the serotype 14 variant of the Spain^{9V}-3 clone and differences between members of the same serogroup in invasive disease potential.**

Invasive disease due to *Streptococcus pneumoniae* (the pneumococcus) is estimated to be responsible for more than one million deaths per annum (13). Despite the heavy toll extracted by the pneumococcus, especially among children and the elderly, this species is commonly carried asymptomatically in the nasopharynx, which represents its primary ecological niche. The pneumococcus is antigenically diverse: 90 different capsular serotypes have been described. Despite this diversity, a relatively small number of serotypes are associated with the majority of invasive disease, although there is substantial variation in the rank order of serotypes that cause disease in different geographic regions (9).

Control of pneumococcal disease is being spearheaded by the conjugate capsular polysaccharide vaccines, which are highly effective at preventing invasive disease due to isolates of the vaccine serotypes (1). In the context of these "imperfect" vaccines, which prevent disease by most but not all of the serotypes that most commonly cause disease, there is considerable interest in an improved understanding of the ability of the different pneumococcal serotypes and strains (clones) to cause invasive disease and the relative contributions to invasiveness of the capsular serotype and the underlying genotype of the strain. Selective pressures applied by vaccination lead to increased carriage of, and exposure to, pneumococci of nonvaccine serotypes (serotype replacement) (14) and may favor the emergence of successful strains, originally of vaccine serotype, that have acquired a nonvaccine capsule through serotype switching (4). The impact of these processes on disease will depend on the ability of the replacing strains to cause disease, which is largely unknown.

An understanding of the relative invasiveness of pneumococcal strains requires a precise procedure for characterizing pneumococci, which is provided by the use of multilocus sequence typing (MLST) (5) and suitable strain collections that allow the prevalence of serotypes and strains which cause invasive disease in a population to be related to the extent of exposure of that population to these pneumococci. Ideally, this would be achieved by comparing the prevalence of each serotype or strain in disease to the extent of their acquisition, but such data are rarely available and difficult to obtain, especially for serotypes or strains with a short mean duration of carriage. The prevalence of strains in asymptomatic carriage has therefore been used as a proxy for acquisition (2).

In this study, we have compared the serotypes and strains of pneumococci that cause invasive disease in children less than 2 years of age in Finland to their prevalence among carriage in Finnish children of the same age.

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MATERIALS AND METHODS

Strains. A total of 224 isolates of *S. pneumoniae* from cases of invasive disease were used in this study. These isolates were cultured from blood or cerebrospinal

fluid of children under the age of 24 months and sent from all laboratories in Finland to the National Pneumococcal Reference Laboratory in Oulu between 1995 and 1999 for serotyping. Serotype was determined by counterimmunoelectrophoresis or by latex agglutination for the neutral serogroups (serotypes) 7 and 14. In the case of uncertain results, serotype was determined by capsular swelling. All antisera for serotyping were purchased from the Statens Seruminstitut, Copenhagen, Denmark. For comparison with isolates from invasive disease, we used a previously published sample of 217 *S. pneumoniae* isolates from carriage in children under 24 months in the Tampere region of Finland (8) collected at three monthly intervals between 1994 and 1996 as part of the Finnish Otitis Media Cohort Study. Repeated isolates of the same sequence type (ST) from the same child were not included.

MLST. MLST was performed as previously described (5). Sequences of each of the seven gene fragments used in the pneumococcal MLST scheme were obtained on both DNA strands by using an Applied Biosystems 3700 capillary sequencer and were edited by using STARS (a free software package available from http://www.mlst.net or http://www.molbiol.ox.ac.uk/~paediat/stars/). Allele assignments were made by using the MLST website (http://spneumoniae.mlst.net). All alleles not already present in the pneumococcal MLST database were verified by resequencing the gene fragment on both strands. Some STs were found to be associated with isolates of more than one serotype. In all such cases, serotype switching was confirmed by repeating the serotyping and MLST of the isolates. A single serotype 6A strain (IO835) was found to have died in storage. DNA from this strain was therefore not available for MLST. Allelic profiles were therefore obtained for 223 strains.

Putative cases of serotype switching were identified as STs associated with more than one serotype. Strains characterized as part of this work were studied in conjunction with previously collected samples of *S. pneumoniae* from Finland together with those in the MLST database. For those examples in which a single example of a novel ST-serotype combination was observed when compared with these datasets, the STs of the isolates were verified by resequencing of the MLST loci and, if necessary, the serotypes were rechecked.

Statistical analysis. The diversity of STs was calculated by using Simpson's index of diversity (*D*). Confidence intervals for *D* were estimated by using the method of Grundmann et al. (7). Odds ratios (ORs) and 95% confidence intervals (CIs) for individual serotypes and STs were calculated relative to all other serotypes (or STs) in the sample, as previously described (9).

TABLE 1. Serotypes recovered from invasive disease in Finland[a]

Serogroup or serotype	No. of invasive isolates (No. of carriage isolates)	OR	95% CI
38	6 (1)	5.94	0.71–49.79
14	40 (11)	**4.07**	2.03–8.17
18C	13 (4)	**3.28**	1.05–10.23
19A	17 (6)	**2.89**	1.12–7.47
7F	15 (6)	2.52	0.96–6.63
4	7 (4)	1.72	0.50–5.95
6B	51 (33)	**1.643**	1.01–2.67
9V	8 (5)	1.57	0.51–4.88
19F	22 (30)	0.68	0.38–1.22
23F	20 (29)	0.64	0.35–1.16
3	1 (2)	0.48	0.04–5.36
10	1 (2)	0.48	0.04–5.36
6A	14 (28)	**0.45**	0.23–0.88
15	2 (5)	0.38	0.07–1.99
22	1 (4)	0.23	0.03–2.15
35F	2 (9)	**0.21**	0.04–0.98
9N	1 (6)	0.16	0.02–1.32
11A	1 (18)	**0.05**	0.01–0.38
8	0 (1)		
12	1 (0)		
21	0 (1)		
25	1 (0)		
28	0 (2)		
34	0 (3)		
18B	0 (1)		
23A	0 (2)		
R	0 (3)		

[a] Numbers of isolates of a given serogroup or type in the invasive and carriage data sets are shown. ORs calculated relative to all other types and 95% CIs calculated from these are also shown. ORs significantly different from 1 are shown in boldface type.

RESULTS

Serotypes and STs recovered from invasive disease in Finland. A total of 224 isolates of *S. pneumoniae* from invasive disease in children <2 years of age (cultured from blood or cerebrospinal fluid) were collected in Finland between 1995 and 1999. Twenty-one different serogroups (serotypes) were identified, the four most common being 6B, 14, 19F, and 23F (Table 1). All of these serotypes are included in the recently introduced seven-valent conjugate vaccine. In total, 71% of isolates from invasive disease were of vaccine serotypes, and an additional 14.8% were of vaccine-related serotypes.

A total of 223 isolates were characterized by MLST, revealing 78 different STs, 26 of which had not been previously recorded in the online pneumococcal database (http://spneumoniae.mlst.net). The most frequent STs were 138, 124, and 191 (serotypes 6B, 14, and 7F, respectively) (Table 2). Each of these STs has been identified as major clones of these serotypes associated with invasive disease in other countries (5). In contrast, ST 482 is a serotype 19A clone that has not previously been reported. The data set also contained isolates of ST 156, the penicillin-resistant Spain^{9V}-3 clone, which was found to mainly express the serotype 14 capsule (7 of 10 isolates, with the remaining 3 comprising a single 19F isolate and 2 9V isolates). The closely related antibiotic-susceptible ST 162 was also present (five 9V isolates, one 19F isolate, and a single 14 isolate).

Of the 224 isolates from invasive disease, 202 were from blood cultures and 22 were from the cerebral spinal fluid. No significant associations were detected between either serotype or ST and the site of isolation of the isolates (data not shown). Use of Simpson's *D* statistic to compare the diversity of STs present in the two samples showed the invasive sample to be significantly less diverse than the carriage sample, with nonoverlapping 95% confidence intervals (invasive sample, $D = 0.038$ [95% CI, 0.027 to 0.048]; carriage sample, $D = 0.019$ [95% CI, 0.014 to 0.024]).

Estimate of odds ratios for serotypes and clones. To assess the invasive disease potential of the serotypes and STs, we compared the invasive isolates with a previously characterized set of strains from nasopharyngeal carriage in children of the same age in Tampere, Finland, collected between 1994 and 1996. Point estimates of the ORs for serogroups (serotypes) and STs are shown in Tables 1 and 2, respectively. The highest OR was associated with serotype 38, although this had wide confidence intervals due to the relatively small numbers of this serotype in the study. Serotypes 14, 18C, 19A, and 6B all had a significantly elevated risk of causing invasive disease. Serotype 6A, in contrast, exhibited a significant association with carriage, as did serotypes 35F and 11A. The majority of invasive disease was due to pneumococci of vaccine serotype, as reflected by a significant association with disease for pooled vaccine serotypes (OR, 2.18; 95% CI, 1.47 to 3.23).

The ORs for the major STs are shown in Table 2. The Spain^{9V}-3 clone, ST 156, was found to be strongly associated

TABLE 2. STs recovered from invasive disease in Finland[a]

ST	No. of invasive isolates (No. of carriage isolates)	Serotype(s) (No. of isolates)	OR	95% CI
156	10 (1 serotype 14)	14 (7), 9V (2), 19F (1)	**10.14**	**1.29–79.91**
496	7 (1)	18C	7	0.85–57.38
482	12 (2)	19A	**6.11**	**1.35–27.65**
393	6 (1)	38	5.97	0.71–50.02
191	14 (4)	7F	**3.57**	**1.16–11.01**
124	20 (8)	14	**2.57**	**1.11–5.98**
138	28 (12)	6B	**2.45**	**1.21–4.96**
483	4 (2)	19F	1.96	0.36–10.83
205	7 (4)	4	1.72	0.50–5.98
162	7 (5 serotype 9V)	9V (5), 19F (1), 14 (1)	1.37	0.43–4.40
309	4 (3)	19F	1.3	0.29–5.89
176	4 (4)	6B	0.97	0.24–3.94
199	6 (8 [3 serogroup 15, 4 serogroup 19A, 1 serogroup 19F])	15 (2), 19A (2), 19F (1), 35F (1)	0.72	0.25–2.12
36	7 (10)	23F	0.67	0.25–1.80
66	2 (4 serotype 9N)	23F (1), 9V (1)	0.48	0.09–2.66
488	2 (4)	6A	0.48	0.09–2.66
37	3 (6)	23F	0.48	0.12–1.94
485	1 (8)	19F	**0.12**	**0.02–0.95**
62	1 (12)	11A	**0.08**	**0.01–0.60**
481	0 (6)	6A		
Other	78 (112)			

[a] Numbers of isolates of given STs present >5 times in the combined invasive and carriage data sets with numbers of isolates of shown serotypes in the invasive data set. ORs calculated relative to all other serotypes and 95% CIs are also shown. ORs significantly different from 1 are shown in boldface type. In the serotypically mixed clones, the serotypes of the carriage isolates are included.

with invasive disease. Significant associations with invasive disease were also found for ST 482 (serotype 19A), ST 191 (a previously defined invasive type 7F clone), ST 124 (a previously defined invasive type 14 clone), and ST 138 (a previously defined invasive type 6B clone). STs 485 and 62 (types 19F and 11A, respectively) displayed significantly reduced invasive disease potential (ORs of <1).

Serotype switching. STs associated with more than one serotype were identified as putative cases of serotype switching. Multiple serotypes associated with a single ST are shown in Table 2 and were found for STs 156, 162, 66, and 199. Among less frequent genotypes, ST 1067 was found as single type 6A and 23F isolates in this data set.

DISCUSSION

The extent to which particular pathogen strains vary in their ability to cause disease is of obvious importance. *S. pneumoniae* is particularly interesting in this regard, as the conjugate vaccines cannot target all of the serotypes and strains that cause disease. While conjugate vaccines are highly effective at preventing invasive disease caused by vaccine serotypes (1), uncertainties exist over their value in preventing less severe disease manifestations such as acute otitis media (8) and the possible consequences of selective pressures imposed on the pneumococcal population by widespread vaccination (14). For invasive disease, the concerns are whether there are nonvaccine serotypes which cause little disease, as there is little exposure to them, but which are relatively invasive and would cause substantial amounts of disease if exposure to these serotypes increased in vaccinated populations as a result of serotype replacement. The invasiveness of a few serotypes excluded from the currently licensed seven-valent vaccine is already well recognized (e.g., serotypes 1 and 5), and coverage of these serotypes will be provided by the higher-valency conjugate vaccines, but for most nonvaccine serotypes, we know very little about their ability to cause invasive disease, nor do we know if the capsule is the primary virulence determinant or if other factors of strains predispose to virulence or more severe disease.

In Finland, the majority of isolates from invasive disease (160 out of 224 [71%]) were of vaccine serotype, and the OR for invasive disease of pooled vaccine serotypes was significantly elevated above unity. As expected, the STs recovered from invasive disease were significantly less diverse than a comparable sample from carriage among children of the same age, suggesting that particular clones are overrepresented in disease compared to carriage.

The ORs for invasive disease obtained for specific serotypes and clones in Finland can be compared to those estimated using a similar study design for pneumococci from Oxford, United Kingdom (2). Because invasive disease is a rare event, it was necessary to include all isolates retrieved from invasive pneumococcal disease in Finland in children less than 24 months of age. The sampling frame for disease is therefore neither perfectly contemporaneous nor contiguous with that for carriage, and this must be accepted as a limitation of the study.

As found in Oxford, there were marked differences in the invasiveness of individual serotypes and clones in Finland, and the results of the two studies were broadly consistent. Excepting serotype 1, which was not represented in the Finnish data sets, all of the serotypes that had ORs >1 in the United Kingdom (types 4, 1, 14, 18C, 7F, 9V, and 19A) also had ORs >1 in Finland. The most marked differences between the two studies were in the estimates of ORs for 6B and 19A, which

were both significantly associated with invasive disease in this study, whereas no strong association was detected previously. Similarly broadly consistent results were further found in a study conducted in Sweden, which compared isolates from invasive disease in all age groups (mainly in individuals >65 years of age) with those carried among children in day care centers (12).

An advantage of carrying out studies in different countries is that the rank order of serotypes that cause invasive disease varies geographically, allowing the invasiveness of additional serotypes to be estimated. Similarly, individual pneumococcal serotypes typically include a number of genetically divergent strains which also may differ between countries, providing comparisons of the invasiveness of different strains of the same serotype. This difference was apparent even between two countries in Europe, since only 6 of the 78 STs that cause invasive disease in Finland were also found in the Oxford data set. The differences in serotypes between Finland and the United Kingdom are relatively minor, but studies in some developing countries may allow estimates of the invasiveness of serotypes that are rarely encountered in industrialized countries.

In Finland, there were sufficient isolates of serotype 11A to show that this nonvaccine serotype has a low OR for invasive disease; only 1 of the 19 isolates of serotype 11A was from disease with an OR of 0.05. Serotype 35F had an OR of 0.2, which was also significantly below 1. These nonvaccine serotypes therefore appear to be poorly invasive. However, the data also demonstrated the invasive potential of some nonvaccine serotypes. For example, serotype 38 was isolated from six cases of disease but was found in only one episode of carriage and had the highest OR in this study, although there were very wide 95% CIs around this point estimate due to the relatively small numbers of isolates. This nonvaccine serotype may be as capable of causing disease as some of the vaccine serotypes, a finding which is consistent with the results of a recent meta-analysis (3). This serotype was among those implicated in serotype replacement in otitis media in two conjugate vaccine trials conducted in Finland (6, 11), suggesting that its prevalence may increase as a result of widespread vaccination, with similarly adverse consequences for invasive disease.

Previously undetected associations with invasive disease were found for serotypes 19A and 6B. Interestingly, the OR for serotype 6B was significantly greater than that for serotype 6A, with nonoverlapping 95% CIs. Similarly, serotype 19A appeared to be more invasive than serotype 19F, although there was a slight overlap in the 95% CIs. An OR which was higher for serotype 6B than 6A was also observed in a recent meta-analysis (3). This result demonstrates that invasive disease potential may vary markedly within serogroups. Whether this variation is due to differences in the structure of the capsule (which are identical for serotypes 6A and 6B, except for the nature of the linkage between the rhamnose and ribitol sugars [10]) or whether it reflects differences in the genotypes of the clones of these serotypes is unclear.

Neither this study nor the study in the United Kingdom detected significant differences in the ORs of genetically distinct clones of the same serotype. However, taking the results of the two studies together, we may compare ORs of three genetically distinct clones of serotype 14. One of these (ST 124) was found in both Oxford and Finland and had an OR that was significantly greater than 1 in both studies. Interestingly, ST 156, which in Finland was of mixed serotype but was predominantly serotype 14, also had a high OR, as did ST 9 in the United Kingdom study. Thus, three distinct serotype 14 clones which are divergent from each other in overall genotype, having different alleles at six or all seven MLST loci, all have high ORs for invasive disease. However, the ORs of some clones with the same serotype were markedly different (e.g., STs 309 and 485), although this was not significant since the confidence intervals overlapped. Because such clones were present in comparatively small numbers, it is possible that this result reflects a real difference that this study was insufficiently able to detect. More studies are required to firmly establish the relative contribution of capsular type to invasiveness compared to that of the overall genotype of pneumococcal strains.

The point estimate of the OR of ST 156 was the highest observed in the Finnish study. ST 156 is the penicillin-resistant Spain^{9V}-3 clone and was derived from the penicillin-susceptible strain ST 162. By MLST, these clones differ at the *ddl* locus, but this change almost certainly occurred when ST 162 acquired penicillin resistance due to the very close genetic linkage between *ddl* and the penicillin-binding protein 2b gene. ST 156 and ST 162 are therefore believed to have essentially the same genotype, and it is interesting that the point estimate for the OR of ST 162, which was most commonly associated with the type 9V capsule, was much lower than that of ST 156, which was mainly serotype 14. Further studies of the ORs of isolates of the same overall genotype, but which differ in serotype, may provide further information on the relative contribution of capsular serotype and overall genotype to the invasiveness of pneumococcal clones. Such studies could be performed using the same study design in a country such as Spain, where both serotypes 9V and 14 variants of the Spain^{9V}-3 clone are commonly encountered.

Several other STs were present in sufficient numbers in Finland to obtain clone-specific ORs. For example, a serotype 19A clone, ST 482, which has as yet been reported only in Finland, showed a highly significant association with invasive disease, whereas ST 485 (serotype 19F) and ST 62 (serotype 11A) were significantly associated with carriage. The serotype 7F clone, ST 191, had an OR of 3.57 and was significantly associated with invasive disease, as was ST 138 (serotype 6B). The latter clone was also present in the Oxford data set, though it was not found to be significantly associated with invasive disease.

The potential for pneumococci to change their capsular locus, and hence their serotype, through recombination has been known for some time (4). Evidence for this occurrence in this data set was limited. While ST 156 and ST 162 were found to express three different capsules, these have all been previously reported. Novel observations were serotypes 23F and 9V isolates of ST 66 and serotypes 19F and 35 isolates of ST 199. The latter ST was also found to express capsular types 15 and 19A in this data set, and the MLST database records further serotype variants of ST199. It is possible that certain lineages of pneumococci are more likely to undergo recombination than others, and further work should investigate this as a matter of urgency, because such strains would be expected to be the most likely to acquire new resistance phenotypes and undergo serotype switching, which could be important following a vaccination campaign.

This work further demonstrates associations between invasive disease and particular serotypes and clones of *S. pneumoniae*. In particular, serotype 14 isolates of the penicillin-resistant Spain^{9V}-3 clone (ST 156) appear to have a marked predilection to cause invasive disease. There is also evidence that rare serotypes such as type 38 may have a previously undetected potential to cause invasive disease and that even very similar serotypes (such as 6A and 6B) may exhibit significant differences in invasiveness, the biological basis of which is obscure. Further work is required to understand the invasiveness of nonvaccine serotypes, although this is problematic due to their rarity in nonvaccinated populations, and to further explore the relative contributions of serotype and genotype to invasiveness. Such studies are important if conjugate vaccines are introduced into childhood vaccination programs, which may be expected to have a major impact upon the pneumococcal population, and to change the serotypes which are transmitted and which may cause disease within vaccinated communities.

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