Comparison of serotyping, pulsed field gel electrophoresis and amplified fragment length polymorphism for typing of *Streptococcus pneumoniae*

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The aim of the present study was to compare serotyping, PFGE and AFLP for typing of *Streptococcus pneumoniae* with regard to discriminatory power, typeability and typing system concordance. Thirty-four isolates from cerebrospinal fluid and 34 time-matched blood culture isolates collected from in-patients at two hospitals in western Norway during the period from January 1994 to May 2002 were included in the study. The discriminatory powers of serotyping, PFGE and AFLP were 0.93, 0.99 and 0.95, respectively. The typeabilities for serotyping, PFGE and AFLP were 1, 1 and 0.99, respectively. A good concordance was shown between all the typing methods. Serotyping would most probably have a higher discriminatory power if further subtyping had been performed. PFGE was more discriminatory than AFLP, and AFLP grouped more-distantly related isolates together. The two typing methods thus provided different information, and therefore both could be useful adjuncts to serotyping for the characterization of *S. pneumoniae*.

Introduction

*Streptococcus pneumoniae* is one of the most common causes of pneumonia, bacteraemia and meningitis (Artz *et al.*, 2003; Catterall, 1999; Mulholland, 1999; Tuomanen, 1999). It is estimated that one to two million adults and at least one million children, mostly in developing countries, die from pneumococcal infections every year (Mulholland, 1999; WHO, 1999). Across Europe also, pneumococcal infections are responsible for considerable morbidity and mortality, particularly in the very young and the elderly (Cartwright, 2002; WHO, 1999).

There is an increasing rate and spread of antibiotic resistance (Cartwright, 2002; Greenwood, 1999; Hall, 1998). *S. pneumoniae* is capable of horizontal transfer of capsule genes, virulence genes and resistance determinants, and there are reported cases of these events occurring (Kalin, 1998; Tuomanen, 1999). There is also the likelihood of pneumococci acquiring antibiotic-resistance determinants from other species (Catterall, 1999; Musher, 1992). The vaccines available are against 7 or 23 capsular serotypes (Hall, 1998; Hausdorff *et al.*, 2000; WHO, 1999) of the overall 90 defined serotypes (Henrichsen, 1995, 1999). There is therefore a risk of emergence and dissemination of strains resistant to antimicrobial agents that are not covered by the vaccines (Tuomanen, 1999).

Serotyping is the traditional phenotypic method used to differentiate pneumococcal strains. Serotypes are grouped together when they share at least one epitope and produce antibodies that cross-react (Henrichsen, 1979). Of the 90 serotypes, 65 belong to 21 different serogroups containing two to five types. Today, serotyping is the standard method used to characterize pneumococci, and the knowledge gained from the type distributions forms the basis of the currently available vaccines.

PFGE, a genotypic method for the evaluation of total chromosomal DNA, has been considered the ‘gold standard’ when typing many micro-organisms and has been employed for typing *S. pneumoniae* (Hall, 1998; Lefevre *et al.*, 1993;
Sa-Leao et al., 2000). AFLP, a genotypic method described in 1995, is based upon the indirect analysis of the whole genome using restriction enzymes (Vos et al., 1995). AFLP is reported to be technically easier than PFGE and has shown good typeability, reproducibility and discriminatory power in the typing of a number of bacterial species including S. pneumoniae (Sugata et al., 2001; van Eldere et al., 1999).

The discriminatory power of a typing scheme is its ability to discriminate between unrelated strains (Hunter & Gaston, 1988; Tenover et al., 1997). Simpson’s index of diversity can be used to objectively assess the discriminatory power of a typing system (Hunter & Gaston, 1988). Typeability is the ability of the typing system to assign a type to each isolate and should ideally be 1, indicating that all isolates are typable (Struelens, 1996). Typing system concordance refers to the concordance of the results by independent typing systems (Struelens, 1996). In the present study, we have compared serotyping, PFGE and AFLP for typing S. pneumoniae.

**Methods**

**Setting.** Haukeland University Hospital is a specialized 1100-bed hospital serving a population of 1000 000 as a referral hospital and 300 000 of this population as an acute-care hospital. The bacteriology laboratory also processes positive cerebrospinal fluid (CSF) and blood cultures from the Deaconess Hospital Haraldsplass, which is an acute-care 186 bed hospital that serves the same population.

**Selection of isolates.** All CSF isolates of S. pneumoniae collected during the period January 1994 to May 2002 at the Department of Microbiology and Immunology, Haukeland University Hospital, Bergen, Norway, were included in the study. Each CSF isolate of S. pneumoniae was matched with an isolate from a blood culture yielding S. pneumoniae from a different patient collected around the same date in order to control for time. During this period, 40 CSF S. pneumoniae isolates were reported, but six of these were unavailable for further analysis. Hence, 34 CSF isolates along with 34 matched blood culture isolates from 67 patients (one patient had two meningitic episodes with a 3 year period) were included in the study. The isolates were stored at –80 °C until they were examined.

**Serotyping.** Pneumococcal serotyping was performed by the Quellung method with antisera from Statens Seruminstitut (Copenhagen, Denmark). The isolates were serogrouped, but were not examined with factor sera to differentiate between types within groups that comprised more than one serovariant (Lund & Henriksen, 1978).

**PFGE.** The isolates were plated onto blood agar and incubated at 37 °C in air with 5% CO₂ overnight. PFGE was performed as described elsewhere (Murray et al., 1990) with modifications (Dahl et al., 1999). DNA was digested using Smal (Promega) and the restricted fragments were resolved using a CHEF-DR III System (Bio-Rad) with the following variables: initial switch time 1 s, final switch time 35 s, total run time 29 h, voltage 6 V cm⁻¹ and inclined angle 120°. Multiple size markers (Lambda Ladder PFGE Marker, New England BioLabs) were applied to the gels. The DNA banding patterns were analysed with BioNumerics version 3.0 (Applied Math) with optimization set at 1-3% and position tolerance set at 0-9%. The Dice coefficient of similarity was calculated, and the unweighed pair group method with arithmetic averages (UPGMA) was used for cluster analysis. A cut-off at 90% similarity of the Dice coefficient was used to indicate identical PFGE types. This corresponds to approximately one band difference, and this degree of similarity also allows for minor technical errors that frequently occur (Salamon et al., 1998). The different PFGE types were used for calculation of Simpson’s index of diversity. A PFGE group was defined as a group of isolates with a similarity of ≥80% of their Dice coefficients (van Eldere et al., 1999).

**AFLP.** DNA was isolated as described elsewhere (Willem et al., 1999), with the addition of a final DNA purification step using ethanol precipitation. AFLP was performed as described elsewhere (Willem et al., 2000) with the following modifications. The DNA was restricted with EcoRI (Promega) and Msel (Invitrogen Life Technologies), and the adapter was constructed by two oligonucleotides (5'-AATTGTAAAAAC-GACGGGCA and 5'-TCTGCGGCTCGTCTTAC) compatible with these endonucleases. Primers EcoRI-A (5'-GGCCGTGGTTTTACAATTCCA) and Msel-0 (5'-ATGACCGGCAGGAAAA) were used for the selective PCR.

The amplification products were run on a DNA sequencer (ABI PRISM 3700, PE Biosystems) for 2.5 h. For this, 1 µl of reaction mixture was mixed with 7 µl distilled water, and subsequently 1 µl of this mixture was diluted with 9 µl formamide containing approximately 0.125 µl GeneScan-500 standard (PE Biosystems). The GeneScan collection software (PE Biosystems) was used to collect data during electrophoresis.

After tracking and extraction of lanes, data were exported to BioNumerics for further analysis. Normalization was done by using the reference positions of the internal DNA size marker GeneScan-500. Fragments ranging in size from 50 to 500 nucleotides were used for comparison. Optimization and position tolerance were set at 0.5%. The Pearson coefficient of similarity of AFLP curves was calculated, and cluster analysis was done by UPGMA. For calculation of Simpson’s index of diversity, isolates with a similarity coefficient of ≥90% were considered to be of the same AFLP type, as described elsewhere (van Eldere et al., 1999). An AFLP group was defined as a group of isolates with a similarity of ≥80% of the Pearson’s correlation coefficient value (van Eldere et al., 1999).

**Comparison of serotyping, PFGE and AFLP.** Simpson’s index of diversity was used to calculate the discriminatory power of the typing methods. The discriminatory power was defined mathematically as the probability that two strains chosen at random from the population would be distinguishable by the typing method (Hunter & Gaston, 1988). A Simpson’s index close to zero indicates that little diversity is shown by the typing method (index of 0 indicates no diversity at all), whereas a Simpson’s index approaching 1 reflects a high diversity, as shown by the typing technique (index of 1 indicates maximum diversity where no isolates are similar). It has been suggested that the Simpson’s index of a good typing system should be greater than 0.95 when testing unrelated isolates (Struelens, 1996). The formula, as suggested by Grundmann et al. (2001), was used to calculate the approximate 95% confidence interval. The typeability of the isolates with the different methods was calculated by the formula recommended by Struelens (1996).

The concordance between PFGE and AFLP was determined using BioNumerics by comparing the values from the similarity matrices of the typing methods that were plotted on an x- and y-graph. Each dot on this graph represents corresponding similarity values for two strains by the typing methods given on the x and y axes. This graph thus gives an indication of the degree of concordance between the two techniques. The Kendall’s τ correlation coefficient between the two methods was also calculated.

In addition, we calculated how many of the isolates that were indistinguishable with one method were also indistinguishable with the other methods. For this calculation, isolates with a similarity of ≥90% of the Dice coefficient were used for PFGE, and isolates with a similarity of ≥90% of the Pearson’s correlation coefficient were used for AFLP. Types containing two or more isolates were included.
Results

Serotyping

Five serotypes and 13 serogroups were identified. Further subtyping of the serogroups was not performed. The most common type was serotype 4, which was present in nine isolates, followed by serotype 1, which was found in eight isolates. The results are presented in Figs 1 and 2.

PFGE

The PFGE banding patterns consisted of 10 to 11 DNA fragments sized between 50 kb and 1000 kb. Among the 68 isolates, 53 types were distinguished using ≥90% similarity of the Dice coefficient as the threshold. When grouping isolates with ≥80% similarity of the Dice coefficient, 16 groups were found. One PFGE group consisted of six isolates, which was the maximum number of isolates in a group, and

Fig. 1. PFGE dendrogram of 68 S. pneumoniae isolates produced following Dice and UPGMA analyses. Sixteen PFGE groups comprising at least two isolates were formed at ≥80% similarity. The distribution of the isolates according to serotype/group and AFLP group are shown. BL, blood culture samples; ND, no data available; SP, spinal fluid samples.

Fig. 2. AFLP dendrogram of 67 S. pneumoniae isolates produced following Pearson and UPGMA analyses. Eight AFLP groups comprising at least two isolates were formed at ≥80% similarity. The distribution of the isolates according to serotype/group and PFGE group are shown. BL, blood culture samples; SP, spinal fluid samples.
nine PFGE groups consisted of two isolates. The dendrogram created by UPGMA, based on the Dice coefficients, is shown in Fig. 1.

**AFLP**

At a level of ≧90% similarity of the Pearson’s coefficient, 28 AFLP types were observed amongst the 67 isolates (Fig. 2). One isolate could not be typed with AFLP because of capillary failure. Upon examination of isolates sharing ≧80% of the restriction fragments, eight groups were discriminated. The largest of these groups contained 16 isolates.

**Comparison of serotyping, PFGE and AFLP**

The discriminatory powers as expressed by Simpson’s index of diversity with 95% confidence intervals (CI) were: for serotyping, 0.93 (CI, 0.92–0.94), for PFGE, 0.99 (CI, 0.98–1.00) and for AFLP, 0.95 (CI, 0.94–0.96). All of the isolates could be typed with serotyping and PFGE. Due to technical failure one isolate could not be typed with AFLP. The typeabilities of the isolates were thus 1 with serotyping and with PFGE, and 0.99 with AFLP. When plotting the pair-wise comparison of isolates typed with PFGE against the pair-wise comparison of isolates typed with AFLP in an x- and y-graph, it was clear that PFGE and AFLP produced results that correlated to some extent, as shown in Fig. 3. Kendall’s τ correlation coefficient was 0.65, which indicates a good concordance.

It was not possible to compare serotyping with the other two methods in this fashion due to the different type of data produced (character data versus fingerprint data). The percentage of isolates that were indistinguishable by one typing method but distinguishable when using the other methods was also calculated. Of the isolates that had the same serotype/serogroup, 66% could be differentiated with PFGE and 17% could be differentiated with AFLP. Of the isolates that were indistinguishable with PFGE, 15% were differentiated with serotyping and 15% were differentiated with AFLP. Of the isolates indistinguishable with AFLP, 5% were differentiated with serotyping and 38% were differentiated with PFGE. These results are presented in Table 1.

**Discussion**

In this study serotyping, PFGE and AFLP were compared with regard to discriminatory power, typeability and typing system concordance in characterizing *S. pneumoniae*. Serotyping had a good discriminatory power (0.93), and all isolates were typable. The isolates belonging to the different serogroups could have been further subtyped, probably resulting in an even higher discriminatory power, as calculated by Simpson’s index of diversity. The discriminatory power of serotyping in this study is therefore most probably a minimal value.

In our study, PFGE showed high discrimination (0.99). This has been previously documented for other organisms when compared to other typing methods (Struelens, 1998). AFLP also had a high discriminatory power (0.95). The choice of restriction enzymes used in the AFLP method is of importance, and when different enzymes were tested (EcoRI/CfoI) and selective primers (EcoRI-A and CfoI-G) the discriminatory power was low, with a Simpson’s index of diversity of 0.86 (data not shown). Therefore, the correct choice of restriction enzymes and selective bases is crucial for high discrimination of strains. It has also been shown that the interpretation of AFLP data (curve-based analysis versus band-based analysis) can also influence the result (Werner et al., 2003).

Only 15% of isolates grouped by PFGE could be differen-

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![Figure 3](https://example.com/fig3.png)

**Fig. 3.** Concordance of stratification by PFGE and AFLP for 67 *S. pneumoniae* isolates. Each dot represents corresponding similarity values for two isolates obtained by the typing methods given on the x and y axes. Kendall’s τ correlation coefficient is 0.65.

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**Table 1. Comparison of differentiation of *S. pneumoniae* isolates by serotyping, PFGE and AFLP**

Numbers indicate the percentages of isolates that were indistinguishable by one typing method (left column) but distinguishable by another method (as indicated by the column headings). For example, 66% and 17% of isolates grouped together with serotyping had a different PFGE type and AFLP type, respectively. Types/groups containing two or more isolates were included.

<table>
<thead>
<tr>
<th>Method</th>
<th>Serotyping</th>
<th>PFGE</th>
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<tr>
<td>Serotyping</td>
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<tr>
<td>PFGE</td>
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<td>AFLP</td>
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tiated with AFLP, whereas 38% of isolates that were grouped with AFLP could be discriminated by PFGE. This difference is due to the fact that generally AFLP gives a higher degree of similarity between the isolates. In a setting where there is no apparent outbreak, and it is of interest to trace less-related strains, this method could be preferable. However, in an outbreak it is important to identify closely related isolates, and it is obvious that the method with the highest discriminatory power would do this most specifically; hence the importance of different typing techniques in different settings. Even without further subtyping it was evident that serotyping was discriminative and only 17% of the isolates within the same serogroup or serotype could be differentiated thus the higher discriminatory power of PFGE.

A comparison of *S. pneumoniae* typing with PFGE and AFLP was undertaken by van Eldere et al. (1999). They reported a general correlation of serotypes with the PFGE and AFLP types. However, they also observed that isolates of serotype 6 did not group together with PFGE or AFLP to the same extent as isolates belonging to other serotypes, which is consistent with our findings. A biological explanation for this is not clear. Hall et al. reported that isolates with highly related PFGE patterns always shared the same serotype (Hall et al., 1996). Lefevre et al. (1993) reported that no correlation between serotypes and PFGE types was present. The lack of correlation between the serotypes and genotypes in some studies might reflect the horizontal transfer of the serotype genes between otherwise genetically unrelated strains. Changes in capsular types due to recombination are well known and have been illustrated by the finding that international epidemic antibiotic-resistant clones may exhibit different serotypes (Dowson & Trzcinski, 2001).

The impact of serotype-based vaccines on the transfer of serotype determinants between strains and on consequent shifts in the prevalence of different serotypes will need to be further investigated. It is therefore of importance to monitor serotypes and clones. It is also important to monitor the spread of *S. pneumoniae* clones resistant to antimicrobial agents. The Pneumococcal Molecular Epidemiology Network (PMEN) (www.sph.emory.edu/PMEN) was established in 1997 with the aim of global surveillance of antibiotic-resistant *S. pneumoniae* and the standardization of nomenclature and classification of resistant clones. The PMEN database includes clones typed with multilocus sequence typing, PFGE, PBP fingerprinting and serotyping.

In the present study PFGE and AFLP were used to elucidate the presence of different strains within the same serotype. Because of the possibilities of horizontal transfer of genes encoding the serotypes, it was clear that PFGE and AFLP data could be of additional value to serotyping. Serotyping, which has stood the test of time and is valuable since type polysaccharides are the vaccine antigens, should, however, still always be done on isolates causing severe disease.

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