Characterization of *Streptococcus pneumoniae* ophthalmic, systemic & commensal isolates by pulsed-field gel electrophoresis & ribotyping

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**Background & objectives**: *Streptococcus pneumoniae* is common in ocular and systemic infections and is a part of normal nasopharyngeal flora. Very few studies regarding genetic analysis of *S. pneumoniae* isolates causing eye infections are available. This study was undertaken to do pulse field gel electrophoresis (PFGE) analysis and ribotyping of *S. pneumoniae* isolates obtained from eye infections, systemic infections and nasopharyngeal flora.

**Methods**: Sixty one well characterized *S. pneumoniae* isolates (38 from ophthalmic infections, 9 from systemic infections and 14 commensals) were characterized using PFGE of the whole genome after SmaI, restriction enzyme digestion and conventional ribotyping using *Escherichia coli* rRNA operon as the probe. Phylogenetic tree was drawn using unweighted pair group method analysis (UPGMA).

**Results**: The 38 *S. pneumoniae* isolates from eye infections belonging to 15 serotypes were placed in to 11 PFGE types and 15 ribotypes. The 9 systemic isolates (7 serotypes) were distributed in 7 PFGE types and 6 ribotypes. The 14 commensal isolates were placed in 11 serotypes, 5 PFGE types and 6 ribotypes. Most of the PFGE types and ribotypes consisting of ocular isolates also contained systemic and commensal isolates.

**Interpretation & conclusions**: Considerable genetic similarity was observed between the isolates from ocular and systemic infections and those colonized in nasopharynx. PFGE analysis could differentiate majority of the isolates according to site of infections. There was a considerable DNA polymorphism within the studied bacterial population.

**Key words** Commensals - ocular infections - PFGE analysis - ribotyping - *S. pneumoniae* - systemic infections

Eye infections by *Streptococcus pneumoniae* include corneal ulcer, dacryocystitis and conjunctivitis in both sporadic and outbreak forms. More often *S. pneumoniae* is associated with pneumonia, bacteraemia.

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meningitis, otitis media and sinusitis. Only a few of the 90 known serotypes are associated with majority of these infections. Moreover, up to 65 per cent of normal population carry *S. pneumoniae* in nasopharyngeal flora.

Both phenotypic and genotypic methods have been developed to characterize the bacterial isolates and to assist in epidemiological investigations. Genetic analysis methods like ribotyping, pulse field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) have shown additional discriminatory power over serotyping.

Genetic analysis along with serotype distribution of *S. pneumoniae* isolates from ophthalmic infections compared to those from systemic infections and nasopharyngeal colonizers are not well known. The present study was undertaken to determine some of the variabilities of genotypes and serotypes among ophthalmic, invasive and colonizing pneumococci.

**Material & Methods**

*Isolation and characterization of the isolates:* Biochemically characterized isolates of *S. pneumoniae* from various ophthalmic infections (dachryocystitis: 28, corneal ulcer: 7, conjunctivitis: 2 and panophthalamitis: 1), 9 isolates from systemic infections (4 with pneumonia, 3 with meningitis, and 2 with septicaemia) and 14 commensal isolates which were serotyped earlier (total 25 serotypes) were included in the study.

**Pulse field gel electrophoresis (PFGE):** Genomic DNA isolation and gel electrophoresis: *S. pneumoniae* DNA embedded in agarose blocks (Bio Rad, USA) was prepared from log phase bacterial culture. The blocks containing genomic DNA were digested with 50 U *SmaI* (Stratagene, USA) restriction enzyme at 25°C overnight. PFGE was performed by the contour-clamped homogeneous electric field method on a CHEF DR-III apparatus (Bio-Rad, Richmond, USA) in 0.5x Tris-Borate-Ethylenediamine tetraacetic acid (TBE) buffer (pH 8.0) in 1 per cent agarose gel for 20 h at 9°C at 6V/cm² with pulse times of 8-35 seconds and an angle of 120° along with a DNA size standard (bacteriophage λ ladder; Bio-Rad, USA). The gels were stained with ethidium bromide (1 μg/ml) for 30 min, and visualized in a gel documentation system (Syngene, Synotics Ltd., USA).

The resulting band patterns were observed visually, and further analyzed using computer software SYNGENE (SYNGENE, Synotics Ltd., USA).

**Ribotyping:** Preparation of DNA probes, Southern blotting, and DNA hybridization.

Genomic DNA was isolated from overnight culture of *S. pneumoniae* by cetyl trimethyl ammonium bromide (CTAB/NaCl) method and digested with restriction enzyme *EcoRI* (Amersham, UK). The fragments were separated by electrophoresis through 0.8 per cent agarose gel (Ambion, USA). Southern hybridization was performed using the radiolabeled *Escherichia coli* rRNA operon as the probe. The 7.5 kb *BamHI* restriction fragment of plasmid pKK 3535, containing the whole rRNA operon was radiolabeled by redi-prime DNA labeling system (Amersham, UK) with [α-32P] dCTP for probe preparation. After hybridization the autoradiographed X-ray films were examined in a gel documentation system and the ribotype patterns were determined using computer software SYNGENE (SYNGENE, Synotics Ltd., USA).

**Computer-assisted analysis of the DNA banding patterns:** A comparison of band patterns was done by the unweighted pair group method (UPGMA) using arithmetic averages and the Dice similarity coefficient (SYNGENE, Synotics Ltd., USA). A tolerance of 0.1 in band positions was applied during the comparison of the fingerprinting patterns. Identical DNA types were defined as those with homologies higher than 80 per cent. A genetic cluster was defined as a genotype that was shared by two or more pneumococcal isolates. The discriminatory index (DI) was determined using Simpson’s index of diversity.

**Results**

**PFGE typing:** The *S. pneumoniae* isolates produced 10 to 19 visible bands in PFGE (Fig. 1). The 61 isolate were distributed in 16 PFGE types: 12 clusters and 4 unique types (Fig. 2). The percentage of similarity between the isolates varied from 51 to 100 per cent and the discriminatory index (DI) was 0.91.

Most of the PFGE types consisted of isolates from all 3 sites (Fig. 2). However, types 5, 7, 10, and 11 contained only one ophthalmic isolate each. The percentage of similarity between ophthalmic isolates varied from 51 to 100 per cent. The 9 systemic isolates were placed in 7 different PFGE types (percentage similarity 71 to 100%). The 14 commensal isolates were placed in 5 types, type 4 containing 8 isolates (percentage similarity 71 to 94%).

**Ribotyping:** The isolates produced 4-8 visual bands in autoradiography (Fig. 3). The 61 isolates were

Table. Serotype distribution and genotypes generated by PFGE and ribotyping of *S. pneumoniae* isolates

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<th>Systemic (n=39)</th>
<th>Commensal (n=14)</th>
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NT, nontypeable serotypes. The ribotypes and PFGE types are numbered the place occupied in the dendogram scale.
Fig. 2. Dendogram of the 61 S. pneumoniae isolates generated by PFGE and ribotyping. PFGE types and ribotypes (numbered from top to bottom), serotypes and sources of isolates are listed. NT, non typeable serotypes. The scale shows the percentage of homology (0-100%) between the isolates.
distributed in 16 ribotypes (Fig. 2); 10 clusters and 6 distinct types; the percentage similarity varied from 32 to 100 per cent; discriminatory index (DI) was 0.905. Most of the ribotypes consisted of isolates from all 3 sources except a few, i.e., ophthalmic isolates in types 3 and 4; and commensal isolates in type 11.

Isolates of ophthalmic infections (38) were distributed in 15 ribotypes (percentage similarity 32 to 100%) (Fig. 2). Six different ribotypes were observed for the 9 isolates form systemic infections (percentage similarity 52 to 100%). The 14 commensal isolates were placed in 6 ribotypes (percentage similarity 55 to 100%).

The relationship between the serotype, PFGE type and ribotype distribution of 61 S. pneumoniae isolates is shown in the Table. Isolates of different serotypes were often placed in the same ribotype or the same PFGE type and vice versa.

**PFGE types vs. ribotypes:** Both the methods distributed the isolates into 16 types each. The discriminatory index of PFGE was slightly higher (DI = 0.91) than ribotyping (DI = 0.905). PFGE typing produced some distinct clusters according to site of isolation i.e. PFGE type 4 contained 8 commensal and 1 ophthalmic isolates, type 5, 10 and 11 contained 5 ophthalmic isolates each, type 7 contained 3 ophthalmic isolates. On the other hand, even though some clustering according to site of isolation was observed in ribotyping, it was markedly less than PFGE typing. We did not observe a single pair of isolates to be genetically identical by both PFGE typing and ribotyping.

**Discussion**

*S. pneumoniae* is one of the predominant bacterial pathogens responsible for eye infections in different parts of the world including India1,2,14. The conjunctival sac and lid margins of the eye harbour a variety of bacteria as normal flora. In previous studies we reported the serotype distributions of these 61 S. pneumoniae isolates from various sites as well as results of BOX-A PCR assay and RAPD analysis to show their genetic relatedness6,9. We characterized these isolates using PFGE and ribotyping in the present study.

Amongst the molecular typing methods PFGE has been used widely to characterize different microorganisms including *S. pneumoniae* because of its high discriminating power, and it is considered to be superior to other molecular typing methods8,14-16. In this study we observed isolates belonging to same serotypes produced different fingerprinting patterns. In contrast, a few isolates of different serotypes produced identical fingerprinting patterns, showing genetic similarity. This indicated that there was considerable DNA polymorphism within the *S. pneumoniae* isolates of the same serotype. Earlier observations suggested that different capsular serotypes can be genetically related but isolates of the same serotypes may show different PFGE patterns17. It was found that PFGE typing could differentiate the isolates to a certain extent according to type and site of infections.

Ribotyping using ribosomal ribonucleic acid (rRNA) gene restriction pattern analysis was the first universal typing method used worldwide18. Isolates of same serotypes were distributed in different ribotypes. Isolates of the same serotype producing different ribotypes has been reported earlier with isolates from invasive disease and pharyngeal carriage19. Members of serotypes 6, 19 and 23, are frequently isolated from the commensal flora and systemic infections in India and other parts of the world14. As these are the serotypes frequently observed among the carriers, there is a further possibility of differentiation into subpopulations within these serotypes detectable by molecular methods like ribotyping.
Between the two genotyping methods, PFGE typing method was more meaningful in terms of discriminating the isolates according to source/site of isolation. Though both the methods distributed the isolated in to 16 genetic types each, clustering of isolates by ribotyping was inconclusive for interpretation. The percentage of genetic similarity among the isolates in ribotyping varied from 32 to 100 per cent whereas it varied from 51 to 100 per cent in PFGE. Both fingerprinting methods were found to be laborious, but PFGE was far more expensive.

The association between the two approaches was highly variable. The isolates belonging to the same ribotype were placed in different PFGE types and vice versa. PFGE typing was rated superior as it could type the isolates according to type and site of infection to some extent.

Isolates from different sources were observed to be in the same genetic cluster by both the genotyping methods showing their genetic relatedness. It is believed that the majority of bacteria cultured from corneal and other eye infections are of the same species that are normally present in the conjunctival sac, on the lids or periocular skin and the adjacent nasal passages. Also it is quite well known that observation frequency of nasopharyngeal colonizer isolates in invasive infections is quite high. Invasive diseases may originate from nasopharyngeal colonizer isolates or could be because of translocation between different sites.

Isolates from ophthalmic infections showing genetic homogeneity with those from the other sources indicated that no specific genotypes were specifically involved in ophthalmic infections. The relatedness among the S. pneumoniae isolates recovered from different sources could be because of translocation between different sites.

In conclusion, to a certain extent PFGE method could segregate the isolates according to site of isolation and type. Isolates from ophthalmic infections showed genetic similarity with others. PFGE method, though more expensive and technically demanding was superior then ribotyping. In future, methods like MLST though further expensive and technically demanding, may provide better options because of their ability to provide more specific sequence based information.

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