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BIOMEDICAL SCIENCE IN BRIEF

Examination of 16S-23S rRNA intergenic spacer region (ISR) heterogeneity in a population of clinical *Streptococcus pneumoniae*- a new laboratory epidemiological genotyping tool to aid outbreak analysis

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Streptococcus pneumoniae (pneumococci) are the most frequent cause of bacterial pneumonia of people of all ages, as well as an important cause of meningitis and otitis media. Although enormous efforts to develop antibiotics and vaccine have been made to overcome pneumococcal infection, infection of *S. pneumoniae* has been a major cause of death especially in developing countries. It is estimated that 100 million people are infected by *S. pneumoniae* every year and 1.6 million people lose their lives. There is also an estimation of child deaths due to pneumococcal infection are 70,000 to 1 million every year around the world [1].

Therefore, it is important to be able to genotype these organisms to strain level, in order to (i) help clinical epidemiology with the typing of strains during an outbreak, in order to determine genetic relatedness amongst outbreak isolates (ii) support basic science studies in estimating the presence of common virulence types, as well as (iii) identify novel strains for incorporation into future vaccine development. To date, examination of heterogeneity with the 16S-23S rRNA intergenic spacer region (ISR) has been poorly examined in pneumococci, as a means of genotyping of these organisms. This 16S-23S rRNA gene sequence spans between the 16S rRNA gene locus and the 23S rRNA gene locus, as detailed in Figure 1. Hence it was the aim of the current study to examine pneumococci from a clinical population, for sequence divergence within the intraspacer region, bridging the 16S and the 23S rDNA gene loci, to ascertain the value of molecular diversity in this region, as a laboratory marker for genotyping different strains of pneumococci.

Seventy-eight isolates of *S. pneumoniae* were obtained from nasal swabs from adult and paediatric patients attending local general practices in Northern Ireland. Only one pneumococcal isolate was used from each patient. These isolates were obtained with informed patient consent as part of a study, which had approval from the Office of Research Ethics Committees (Northern Ireland) [ORECNI]. All samples were plated onto selective Mitis-Salivarius agar (cat no: 229810, Becton Dickinson Ltd., Oxford, UK) containing 1% [w/v] tellurite solution and were incubated for 48 h at 37 °C under microaerophilic conditions in a CO_2 incubator regulated at 5%[v/v] CO_2. Following incubation, presumptive *S. pneumoniae* isolates were subcultured onto Columbia Blood agar (CM0331 Oxoid Ltd., Basingstoke, UK), supplemented with 5% (v/v) defibrinated horse blood for 24 h at 37 °C under microaerophilic conditions, as detailed above. The species of all presumptive pneumococcal isolates was confirmed phenotypically by optochin susceptibility testing, as well as bile solubility testing. In addition, all isolates were confirmed molecularly through sequence analysis of the *rpnB* and 16S rRNA gene loci [2].

Bacterial isolates were subcultured on Columbia blood agar supplemented with 5% (v/v) Horse Blood (Oxoid Ltd. Basingstoke, UK), for 24 h at 37 °C under microaerophilic conditions. Bacterial genomic DNA was extracted from a few colonies of each isolate, by employment of the Roche High Purity PCR Template Preparation Kit (Roche Diagnostics Ltd., Sussex, UK), in accordance with the manufacturer's instructions. Extracted DNA was stored at –20 °C prior to PCR amplification.

The 16S-23S rRNA gene operon was amplified in each isolate using a previously described method for viridans group streptococci (VGS), using the primers, 13BF (5′ – GTGAATACGTTCCCGGGCCT - 3′) [3] and 6R (5′- GGGTTYCCCCRTTCRGAAAT – 3′) [4]. Following amplification, PCR products were visualised on 1.5% (w/v) agarose gels in 0.5 × TBE buffer followed by staining with ethidium bromide (0.5 µg/ml) and UV illumination

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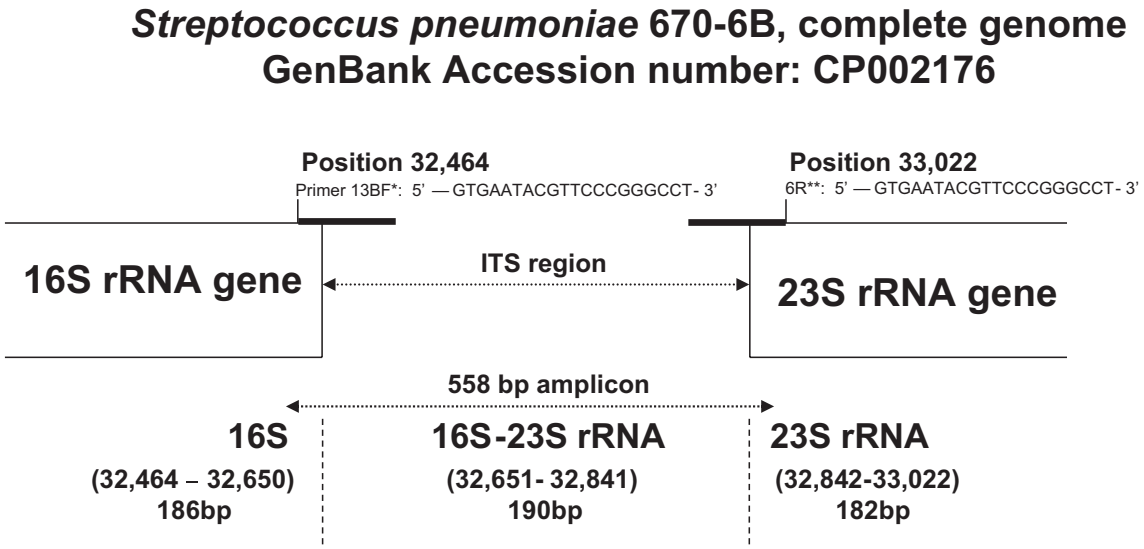

Figure 1. Description of the ribosomal RNA gene arrangements in *Streptococcus pneumoniae*, including location of PCR primer sequences used and product length.

Table 1. Frequency and distribution of 16S-23S genotypes obtained from a population of 78 pneumococci. A representative sequence of each genotypes is shown at the GenBank Accession number cited.

Genotype	Number of isolates	Isolate reference	Submitted GenBank Accession number
A	29	5, 7, 11, 13, 14, 15, 16, 19, 20, 24, 25, 28, 32, 33, 35, 36, 40, 42, 44, 48, 52, 58, 61, 62, 70, 73, 75, 77, 78	JN705728
B	20	18, 26, 29, 31, 39, 41, 43, 45, 47, 49, 51, 53, 59, 60, 65, 66, 67, 72, 79, 80	JN705729
C	5	6, 30, 37, 57, 68	JN705730
D	2	1, 3	JN705731
E	2	63, 69	JN705732
F	2	8, 22	JN705733
G	1	2	JN705734
H	1	9	JN705735
I	1	23	JN705736
J	1	54	JN705737
K	1	17	JN705738
L	1	38	JN705739
M	1	76	JN705740
N	1	34	JN705741
O	1	64	JN705742
P	1	71	JN705743
Q	1	56	JN705744
R	1	21	JN705745
S	1	50	JN705746
T	1	55	JN705747
U	1	4	JN705748
V	1	10	JN705749
W	1	27	JN705750
X	1	74	JN705751
Y	1	12	JN705752
Total = 25 genotypes	78 isolates	78 isolates	25 sequence types

with a gel image analysis system (UVP Products Ltd., Cambridge, UK). Amplicons for sequencing were purified with a QIAquick PCR purification kit (QIAGEN Ltd., West Sussex, UK), according to manufacturer's instructions and resulting PCR amplicons of approximately 558 bp were sequenced in both directions, using the BigDye Terminator Cycle Sequencing kit and ABI 3100 Genetic Analyser (Applied Biosystems). Sequencing analysis was carried out with the GENETYX (Genetyx corporation, Tokyo, Japan). Resulting sequence chromotograms were carefully checked manually in both the forward and reverse directions and any ambiguities resolved by comparison of forward and reverse sequence data. Following deletion of the flanking 16S rRNA and 23S rRNA regions (186 bp and 182 bp, respectively), genotypes were assigned arbitrarily on the remaining 190 bp 16S-23S rRNA region, based on either (i) at least one base substitution, or (ii) at least one base insertion or (iii) at least one base deletion.

The complete 16S-23S rRNA region was successfully amplified in all 78 *S. pneumoniae* isolates examined. A representative sequence of each genotype was subsequently submitted to GenBank, with Accession numbers shown in Table 1. The resulting 16S-23S rRNA sequences

were aligned and a dendogram produced, displaying the phylogenetic relationships between all 78 isolates examined (Table 1). Twenty-five 16S-23S genotypes (Genotype A – Genotype Y) were identified amongst the 78 isolates, with two predominant types, i.e. Genotype A and Genotype B, with 28 and 20 members, respectively. These two genotypes accounted for 61.5% of the total pneumococci examined, with the remaining isolates clustering into 19 genotypes (Genotypes G–Y) containing only one member (Table 1).

Genetic variation in the intraspacer region of the 16-23S rRNA gene loci is an attractive marker for bacteriological strain typing, thereby allowing the differentiation of strains from each other. All bacteria inherently possess such rRNA genetic machinery, therefore, it is not necessary to need to employ species-specific PCR primers, thereby circumventing the need to have prior sequence knowledge. This molecular technique has been used with other bacterial species, including staphylococci [5] and *Legionella* [6]. The need to sequence these specific gene loci is pivotal to the success of such a genotyping scheme. DNA sequencing is now becoming cheaper, as well as more available to NHS laboratories, either onsite through a genomic core facility, as operated by certain hospitals in partnership with universities, or via post, to a third party private sequencing.

In conclusion, the complete 16S-23S rRNA region in 78 *Streptococcus pneumoniae* (*S. pneumoniae*) isolates were examined through PCR amplification and sequencing to determine the degree of sequence heterogeneity within this internal transcibed spacer (ISR) region. Twenty-five 16S-23S genotypes (Genotype A – Genotype Y) were identified amongst the 78 isolates, with two predominant types, i.e. Genotype A and Genotype B, with 28 and 20 members, respectively. These two genotypes accounted for 61.5% of the total pneumococci examined, with the remaining isolates clustered into 19 genotypes (Genotypes G–Y) containing only one member. This study showed that this ITS region in community-acquired isolates of pneumococci is variable and not totally conserved. Further comparative work is now required to determine its suitability as an epidemiological genotyping scheme.

This work represents an advance in biomedical science because it describes a novel genotyping method to aid in outbreak epidemiological analysis for *Streptococcus pneumoniae* in Clinical Microbiology laboratories.

Disclosure statement

No potential conflict of interest was reported by the authors.

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