Clinical streptococcal isolates, distinct from *Streptococcus pneumoniae*, but containing the β-glucosyltransferase *tts* gene and expressing serotype 37 capsular polysaccharide

Carmen L SheppardCorresp., 1, Georgia Kapatai 1, Karen Broughton 1, Ulf Schaefer 2, Matthew Hannah 3, David J Litt 1, Norman K Fry 1

1 Respiratory and Vaccine Preventable Bacteria Reference Unit, Public Health England, London, United Kingdom
2 Infectious Disease Informatics/Bioinformatics, Public Health England, London, United Kingdom
3 Virus Reference Department, Public Health England, London, United Kingdom

Corresponding Author: Carmen L Sheppard
Email address: Carmen.Sheppard@phe.gov.uk

The major virulence factor of the pneumococcus, and target for conjugate vaccines, is the polysaccharide capsule, which is usually encoded by the highly variable *cps* locus. Serotype 37 is an unusual pneumococcal type in which the single β-glucosyltransferase gene responsible for serotype capsule production (*tts*) is located outside of the capsular operon region. Using a previously described automated whole genome sequence (WGS)-based serotyping bioinformatics tool, PneumoCaT, we identified and investigated seven clinical isolates (three from blood cultures) of non-pneumococcal streptococci containing a highly homologous *tts* and included them in a study panel of 20 isolates which included a 11 further clinical isolates of *S. pneumoniae* serotype 37, a reference strain of serotype 37 and the *S. pseudopneumoniae* type strain BAA 960T. The seven non-pneumococcal isolates generated novel alleles at all pneumococcal MLST loci and gave low percentage similarity (<45%) to *S. pneumoniae* or *S. pseudopneumoniae* species by comparison of short sequence patterns in genomic data (kmer analysis). The *S. pseudopneumoniae* BAA-960T isolate generated two novel alleles in the MLST and gave a high similarity (>99%) to the reference sequence for BAA-960T. Twelve isolates gave high similarity (>77%) to the *Streptococcus pneumoniae* 5652-06 serotype 19A reference genome sequence and had previously reported MLST alleles. Each of the seven clinical non-pneumococcal strains and all of the 12 *S. pneumoniae* possessed a β-glycosyltransferase gene (*tts*) with >95% similarity to the pneumococcal *tts* reference DNA sequence with 20-22 non-synonymous SNPs. All, but two strains in which the *tts* gene was detected gave positive reactions for serotype 37 in slide agglutination tests with serotype 37 typing sera. Phylogenetic analysis using both SNP and MLST data showed distinct clades corresponding to strains identified as pneumococcus or non-pneumococcus by kmer WGS analysis. Extended kmer database analysis and ribosomal MLST placed the non-pneumococcal isolates within the *S. mitis*
group. Biochemical and bile solubility assays showed differences between the unusual isolates and *S. pneumoniae*. All isolates had detectable pneumolysin (*ply*) genes, but only those that identified as pneumococcus contained the genes for autolysin (*lytA*) or the ABC transporter lipoprotein A (*piaA*) with >80% coverage and >95% similarity. Here we report the existence of a novel group of strains distinct from *S. pneumoniae*, but which can express a pneumococcal serotype 37 capsular polysaccharide which can be associated with clinical disease.
Clinical streptococcal isolates, distinct from *Streptococcus pneumoniae*, but containing the β-glucosyltransferase *tts* gene and expressing serotype 37 capsular polysaccharide.

Carmen L. Sheppard¹, Georgia Kapatai¹, Karen Broughton¹, Ulf Schaefer², Matthew Hannah³, David J. Litt¹, Norman K. Fry¹

¹: Respiratory and Vaccine Preventable Bacteria Reference Unit, Public Health England - National Infection Service, 61 Colindale Avenue, London, NW9 5EQ.

²: Infectious Disease Informatics/Bioinformatics, Public Health England, 61 Colindale Avenue, London, NW9 5EQ.

³: Virus Reference Department, Public Health England, 61 Colindale Avenue, London, NW9 5EQ.

Corresponding Author:

Carmen Sheppard¹

Email address: Carmen.Sheppard@phe.gov.uk
ABSTRACT

The major virulence factor of the pneumococcus, and target for conjugate vaccines, is the polysaccharide capsule, which is usually encoded by the highly variable cps locus. Serotype 37 is an unusual pneumococcal type in which the single β-glucosyltransferase gene responsible for serotype capsule production (tts) is located outside of the capsular operon region. Using a previously described automated whole genome sequence (WGS)-based serotyping bioinformatics tool, PneumoCaT, we identified and investigated seven clinical isolates (three from blood cultures) of non-pneumococcal streptococci containing a highly homologous tts and included them in a study panel of 20 isolates which included a 11 further clinical isolates of S. pneumoniae serotype 37, a reference strain of serotype 37 and the S. pseudopneumoniae type strain BAA 960T.

The seven non-pneumococcal isolates generated novel alleles at all pneumococcal MLST loci and gave low percentage similarity (<45%) to S. pneumoniae or S. pseudopneumoniae species by comparison of short sequence patterns in genomic data (kmer analysis). The S. pseudopneumoniae BAA-960T isolate generated two novel alleles in the MLST and gave a high similarity (>99%) to the reference sequence for BAA-960T. Twelve isolates gave high similarity (>77%) to the Streptococcus pneumoniae 5652-06 serotype 19A reference genome sequence and had previously reported MLST alleles. Each of the seven clinical non-pneumococcal strains and all of the 12 S. pneumoniae possessed a β-glycosyltransferase gene (tts) with >95% similarity to the pneumococcal tts reference DNA sequence with 20-22 non-synonymous SNPs.
All, but two strains in which the \textit{tts} gene was detected gave positive reactions for serotype 37 in slide agglutination tests with serotype 37 typing sera. Phylogenetic analysis using both SNP and MLST data showed distinct clades corresponding to strains identified as pneumococcus or non-pneumococcus by kmer WGS analysis. Extended kmer database analysis and ribosomal MLST placed the non-pneumococcal isolates within the \textit{S. mitis} group. Biochemical and bile solubility assays showed differences between the unusual isolates and \textit{S. pneumoniae}. All isolates had detectable pneumolysin (\textit{ply}) genes, but only those that identified as pneumococcus contained the genes for autolysin (\textit{lytA}) or the ABC transporter lipoprotein A (\textit{piaA}) with >80% coverage and >95% similarity.

Here we report the existence of a novel group of strains distinct from \textit{S. pneumoniae}, but which can express a pneumococcal serotype 37 capsular polysaccharide which can be associated with clinical disease.

\section*{INTRODUCTION}

The polysaccharide capsule of \textit{Streptococcus pneumoniae} (pneumococcus) is an essential virulence factor (Nelson \textit{et al.}, 2007) and a distinguishing characteristic of the species compared to other closely related, non-encapsulated streptococci such as \textit{S. pseudopneumoniae}, \textit{S. oralis} and the \textit{S. mitis} group streptococci. These other non-encapsulated species predominantly cause non-invasive disease, but can occasionally cause invasive diseases such as endocarditis and other
infections in immunocompromised patients. They may also contain pneumococcus-like virulence genes such as autolysin (lytA) and pneumolysin (ply) leading to their potential misidentification as *S. pneumoniae* (Whatmore *et al.*, 2000; Balsalobre *et al.*, 2006; Johnston *et al.*, 2010). The capsule of the pneumococcus plays a significant role in its pathogenesis and pneumococcal disease is a major global public health issue and cause of morbidity and mortality in young children and adults, in both developed and developing countries. The range of diseases caused by pneumococci includes severe manifestations, e.g., pneumonia, meningitis and bacteraemia, to less serious ones, such as, otitis media, sinusitis and bronchitis (http://www.who.int/immunization/topics/pneumococcal_disease/en/; 5). The capsule is also the target of all current licenced vaccines for *S. pneumoniae*, and the introduction of conjugate vaccines to the most common capsular types has led to a dramatic reduction in circulating vaccine serotypes and an increase in non-vaccine serotype disease (Waight *et al.*, 2015).

In 90 of the 92 serotypes, defined in the Danish system by the commercial typing sera manufacturer Staten Serum Institute (SSI), Copenhagen, Denmark (http://www.ssi.dk/), the capsular polysaccharide is produced via the *wzx*-*wzy* dependent biosynthetic pathway, using a cluster of genes in an operon located between the *dexB* and *aliA* genes in the pneumococcal genome (Bentley *et al.*, 2006). Serotypes 3 and 37 are unusual serotypes that utilise the synthase pathway for capsule production. In the synthase pathway, a single synthase gene produces an enzyme located in the cell membrane, which assembles and extrudes the simple polysaccharide chains (Yother, 2011). Serotype 37 pneumococcus has the gene for capsular polysaccharide production (*tts*) located outside of the capsular operon region (Llull *et al.*, 1999), whereas in
serotype 3, although a single gene (*wchE*) is also responsible, it is located within the regular

capsular operon location (Bentley *et al.*, 2006).

Serotype 37 pneumococci have a redundant, serotype 33F-like capsular operon in the region
between *dexB* and *aliA*, which does not play a part in the production of serotype 37 capsular
polysaccharide (Llull *et al.*, 1999). Llull *et al* described the transformation of other
pneumococcal serotypes to a binary capsule type (that is expression of serotype 37 plus another
serotype) by insertion of the *tts* gene into other serotypes of pneumococcus. In further work,
authors from the same laboratory demonstrated expression of the serotype 37 polysaccharide in
other Gram-positive bacterial species, *S. oralis*, *S. gordonii* and *Bacillus subtilis* by laboratory
transformation with plasmids containing the *tts* gene (Llull, Garcia and Lopez, 2001).

During development, validation and use of a bioinformatics pipeline for determining serotype in
*S. pneumoniae* from whole genome sequencing (WGS) data, PneumoCaT (Kapatai *et al.*, 2016),
we discovered a number of isolates that were reported as *S. pneumoniae* serotype 37 using phenotypic
methodology, but were subsequently identified as non-pneumococcal Streptococcus spp. by whole
genome kmer analysis. They all contained a gene with high similarity to the serotype 37 capsule
production gene, *tts*. We believe this to be the first description of non-pneumococcal isolates
containing a *tts* β-glycosyltransferase gene and expressing serotype 37 polysaccharide,
associated with clinical disease.

We found a total of 7 non-pneumococcal streptococcal isolates identified by genomic analysis
that each contained the *tts* gene. This study describes the characterisation of these isolates by
WGS analysis and phenotypic methods.
MATERIALS AND METHODS

Study Isolates

We created a study panel of 20 isolates, comprising 18 clinical isolates which were shown to contain the tts gene by PneumoCaT WGS analysis (described below), the serotype 37 S. pneumoniae type strain (SSI-37) and the S. pseudopneumoniae type strain (BAA-960^T). This panel of isolates was subjected to full characterisation using both phenotypic and genotypic methods.

Genomic data from an additional 17 S. mitis (N=5), S. pseudopneumoniae (N=6) and S. pneumoniae (N=6, serotypes 23B, 14, 3, 22F, 8 and non-typeable) strains identified by kmerID, were also used for phylogenetic comparisons only, referred to as “extra isolates” in this manuscript.

Clinical isolates were received as part of the Public Health England (PHE) surveillance of pneumococcal infections in which referral of invasive S. pneumoniae (i.e., from blood and CSF) is currently requested from hospital laboratories in England and Wales to the Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU), London for serotyping. In addition, some pneumococci from non-sterile sites (e.g., sputum isolates) are also referred for antibiotic resistance testing and pass through RVPBRU for confirmation of ID and serotyping before being sent to the Antimicrobial Resistance and Healthcare Associated Infections Reference Unit for antibiotic resistance testing.
Isolates of *S. pseudopneumoniae* BAA-960\textsuperscript{T} and the *S. pneumoniae* serotype 37 reference strain (SSI-37) were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA and Statens Serum Institut (SSI) Denmark, respectively.

All sequences from the study isolates that were identified as pneumococcus and one of the non-pneumococcal isolates are available on the European Nucleotide Archive as part of our previous work (Kapatai *et al.*, 2016), under project PRJEB14267, all other sequences used in this study are submitted to ENA under project number PRJEB20507, see Supplementary Table 1.

**Phenotypic methods**

Isolates were grown on Columbia blood agar (Oxoid, UK) and had been putatively identified as *S. pneumoniae* by demonstration of optochin sensitivity using 5 µg optochin disc (Oxoid-Thermofisher, Basingstoke, UK). For phenotypic serotyping, isolates were grown for four hours or overnight in 5 ml MAST Todd Hewitt broth (PHE Media Services) at 35°C with 5% CO\textsubscript{2}, centrifuged at 453 x g for 30 min, the supernatant removed, the cell pellet re-suspended in a small residual volume of broth and serotyped by slide agglutination tests with standard antisera (SSI, Copenhagen, Denmark). The cell suspensions were also mixed with typing sera on a slide and methylene blue (SIGMA UK) was added. These were visualised under a light microscope using a 100x oil immersion objective.

Additional biochemical testing was performed using API Rapid ID Strep 32 (bioMérieux, Basingstoke, UK) according to the manufacturer’s instructions. Standard deoxycholate bile solubility test was performed by culture of the organism in duplicate test tubes containing 5 ml Todd-Hewitt broth. After centrifugation at 453 x g for 30 min the supernatants were discarded,
cell pellets resuspended in 2.5 ml of phosphate-buffered saline (PBS), and 0.5 ml of 10% sodium deoxycholate added to one of the tubes (the other being the control). These were incubated at room temperature for 30 min. Complete lysis within 30 min of incubation in the tube containing the deoxycholate indicated a soluble result and incomplete lysis was scored as “indeterminate” (Keith et al., 2006).

**Electron Microscopy**

Electron microscopy using colony lifts was performed to visualise the general morphological characteristics of selected isolates. In this technique, 400 mesh Copper EM grids (Agar Scientific), coated in-house with pioloform (Agar Scientific) and carbon were placed directly onto the surface of bacterial colonies growing on blood agar and light pressure applied. After 1-2 min resting on the colonies, the EM grids were carefully removed and placed (cell-side-down) onto a drop of 3% (w/v) paraformaldehyde (PFA) in PBS. After 15 min incubation on the PFA, the grid was removed, its surface washed twice with deionized water and the bacterial cells negatively stained with 1.5% (w/v) phosphotungstic acid (Taab Laboratories). After drying the EM grids were examined in a JEM-1400 transmission electron microscope (JEOL UK) and images acquired using an AMT XR60 digital camera (Deben UK).

**DNA Extraction and sequencing**

Bacterial growth was harvested from the agar plates and pre-lysed by the recommended method for Gram-negative bacteria (Qiagen, Manchester, UK), which is effective for pneumococcus and closely related streptococci. DNA was extracted from the lysates using a QIAsymphony SP
automated instrument and a QIAsymphony DSP DNA Mini Kit (Qiagen), using the tissue
extraction protocol. DNA concentrations were measured using the Quant-IT Broad Range
dsDNA Kit (Life Technologies, Paisley, UK) and GloMax® 96 Microplate Luminometer
(Promega, Southampton, UK). WGS was performed using Illumina methodology by the PHE
Genomic Services Delivery Unit (GSDU, Colindale, UK). The resulting data were automatically
analysed using an in-house bioinformatics pipeline for *S. pneumoniae*.

**PHE Reference Bioinformatics workflow for *S. pneumoniae***

Casava 1.8.2 (Illumina inc. San Diego, CA,USA) was used to deplex the samples and FASTQ
reads were processed with Trimmomatic to remove adapters and bases from the trailing end that
fall below a PHRED score of 30. K-mer identification software (kmerID,
https://github.com/phe-bioinformatics/kmerid) was used to compare the sequence reads with a
panel of curated NCBI RefSeq (https://www.ncbi.nlm.nih.gov/refseq/) genomes to identify the
species. KmerID measures the identity between a reference genome and a set of WGS reads by
determining the percentage of 18-mers in the reference genome that also occur at least twice in
the WGS reads. Thus, each genomic sequence in the *S. pneumoniae* bioinformatics workflow
was compared to 1769 reference genomes representing 59 pathogenic and commensal bacterial
genera obtained from RefSeq. The closest percentage match was identified, and provided initial
confirmation of the species. This step also identified isolates containing more than one species of
bacteria (i.e., mixed cultures) and any organisms misidentified as *S. pneumoniae* by the sending
laboratory. Further analysis (for MLST type and serotype) using the automated *S. pneumoniae*
workflow continues only if *S. pneumoniae* was identified in the top five hits using the kmerID.
*Streptococcus pneumoniae* MLST data were derived from the genomic data using the Metric-Oriented Sequence Typing software ([Tewolde et al., 2016](https://github.com/phe-bioinformatics/MOST)) using allele definitions downloaded from PubMLST during the period Oct 2014 to December 2016. WGS serotype was derived from the data using a version of PneumoCaT ([Kapatai et al., 2016](https://github.com/phe-bioinformatics/PneumoCaT)) incorporated as the final analytic stage of the in-house *S. pneumoniae* WGS bioinformatics pipeline. Specific to this study, the PneumoCaT workflow initially mapped reads from the subject to a reference database containing capsular operon sequences for all serotypes, including serogroup 33 capsular operon sequences and the *tts* sequence. Due to the presence of the pseudo-33F capsular operon, pneumococcal isolates give >90% coverage for the 33F and 33A operon as well as for the *tts* gene. However, serotype 37 is determined by the presence of the *tts* gene (with >90% coverage and minimum depth of 5 reads per bp).

**Further genomic analysis methods**

**Analysis of the detected *tts* sequences**

As PneumoCaT only provides coverage statistics for the *tts* genes detected, the NCBI BLAST website ([https://blast.ncbi.nlm.nih.gov](https://blast.ncbi.nlm.nih.gov)) was used to assist extraction of the *tts* sequence from the contigs and query the BLAST nucleotide collection database (nr/nt) and the BLAST protein collection database (pr) using algorithms blastn and blastp, respectively.

The amino acid explorer tools at the NCBI website ([https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi](https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi)) were used to assess the amino-acid substitutions observed between the study sequences and the reference TTS sequence.
and assess their likelihood of occurring in a homologous protein using the BLOSUM62 matrix (Henikoff and Henikoff, 1992).

Evaluation of kmerID percentage similarity threshold for streptococcal species determination

KmerID uses the percentage of 18-mers in a given reference genome that are also present in a given set of WGS sequencing reads at least twice. In order to determine a percentage similarity threshold for the kmerID method for a positive identification of a species the Jaccard index of set similarity (JI) was used to investigate the relationship of 798 genomes belonging to 58 different streptococcal species to each other. Given two streptococcal genomes and the respective sets of 18-mers in these two genomes represented as A and B, the JI is calculated as the size of the intersection between A and B divided by the size of the union of A and B. For the 18 streptococcal species for which a minimum of 5 genomes are available, we compared the intra-species JIs with the inter-species indices and thus determined threshold values at which species can be reliably identified.

For this purpose, we iterated over a range of JI cut-off values and calculated the Matthew’s correlation coefficients (MCCs) based on the resulting confusion matrices. Areas in which the MCC reaches 1.0 for a given species denote threshold values at and above which this species can be reliably distinguished from other species.

In order to assess the population structure of the reference sequences a Neighbour Joining tree was constructed for 798 streptococcal genomes using the JI as a measure of similarity.
Extended streptococcal kmerID analysis

Sequence data from all the isolates in the study were re-analysed by kmerID as described above using an extended streptococcal reference database containing 798 streptococcal genomes of 58 different species (referred to as “extended streptococcal kmerID”, list of species shown in Supplementary Table 2).

Ribosomal MLST

Genomes were assembled using SPAdes version 3.8.5 (Bankevich et al. 2012) and the resulting contigs used to query the 53 allele ribosomal MLST database (http://pubmlst.org/rmlst/) to determine rps alleles and obtain a ribosomal sequence type (rST) and information (including species identification) of isolates submitted to the database if an exact rST profile match was achieved. The database was also queried for speciation using the “Identify Species” link at the PubMLST rMLST website (http://pubmlst.org/rmlst/ accessed April 2016), in which the alleles are compared one by one, (rpsA-rpmJ) and the first rps allele sequence with an exact match in the database is reported along with the species of the isolate that contains the matching allele (Jolley et al., 2012).

Whole Genome SNP analysis

Genomic reads from the study set of isolates plus the extra sequences from S. pneumoniae isolates of different serotypes (serotype 23B, 8, 3, 14 and nontypeable) and S. pseudopneumoniae sequenced at PHE were mapped to the acapsular S. pneumoniae R6 reference
sequence (NCBI accession number NC_003098) using BWA-MEM version 0.7.12 (Li and Durbin, 2009). Variants were called using GATK 2.6.5 (McKenna et al., 2010). Variants were then filtered to retain high quality SNPs based on the following conditions: depth of coverage (DP) ≥5, AD ratio (ratio between variant base and alternative bases) ≥0.8, Mapping Quality (MQ) ≥30, ratio of reads with MQ0 to total number of reads ≤0.05. All positions that fulfilled the filtering criteria in >90% of the samples were joined to produce a multiple fasta format file where the sequence for each strain consists of the concatenated variants. This file was used as an input to generate a maximum likelihood (ML) tree using RAxML (Stamatakis, 2014) with the following parameters –m (substitutionModel) GTRCAT –b (bootstrapRandomNumberSeed) 12345 -# (numberOfRuns) 1000.

Distance matrices were constructed and group analysis was performed using MEGA 7 Software (Kumar, Stecher and Tamura, 2016).

Multi-Locus Sequence Analysis

Multi-Locus Sequence Analysis (MLSA) (Hanage, Fraser and Spratt, 2006; Glaeser and Kämpfer, 2015) was performed by extracting the MLST allele sequences for aroE, gdh, gki, recP, spi and xpt from the pileup files created during the MLST analysis using MOST and concatenated. The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992) using MEGA 7 (Kumar, Stecher and Tamura, 2016) to construct a minimum evolution tree using 100 bootstraps. The trees also included concatenated sequences from the small panel of extra S. pneumoniae, S. mitis and S. pseudopneumoniae isolates obtained from the PHE collection for further comparison.
Detection of \textit{lytA}, \textit{ply} and \textit{piaA} genes

The widely used pneumococcal PCR target genes \textit{lytA} and \textit{ply} together with the additional gene \textit{piaA}, were detected in the study set using a mapping-based approach using published bowtie 2 and Samtools software (Langmead, 2010), GenBank KP110770, HG531769 and AF338658.1:111-1130 sequences respectively were used as references and a cut-off of 80% coverage and 95% identity were used to indicate a positive gene detection in this study.

RESULTS

Phenotypic results

The panel of 20 isolates was characterised using a variety of methods (Tables 1 and 2). A major and immediately obvious phenotypic difference between the pneumococcal and non-pneumococcal isolates in this study was that the pneumococcal isolates had a very mucoid appearance on the blood agar plates. In contrast, the seven clinical isolates that were non-pneumococcus (by kmerID) had small, non-mucoid colonies. However, they still demonstrated a glistening appearance and a smoother consistency compared to the dry, rough colonies of the \textit{S. pseudopneumoniae} type strain (BAA-960\textsuperscript{T}).

Further biochemical analysis by bile solubility, API Strep 32 and serotyping gave the results shown in Table 1. The non-pneumococcal clinical isolates gave variable results in the API biochemical test, with most giving a biochemical profile result consistent with \textit{S. oralis} (N = 4), \textit{S. mitis} (N = 2) and \textit{S. pneumoniae} (N = 1), they also gave resistant (N=1) or indeterminate (incomplete clearing, N=6) results with the bile solubility test. All the kmerID \textit{S. pneumoniae}
serotype 37 strains were also identified as *S. pneumoniae* by API 32 Strep and gave soluble results with the bile solubility test. The BAA-960$^T$ *S. pseudopneumoniae* type strain identified as *S. oralis* by API strep 32.

We were able to confirm serotype 37 by slide agglutination in five of the seven non-pneumococcal isolates (Table 1). Observation under the microscope (x100 oil immersion) also showed agglutination of the cells when type 37 serum (SSI) was applied to a bacterial suspension. Due to the very mucoid, non-cell wall associated, nature of the capsule in serotype 37 (and serotype 3), it is not usually possible to observe capsular swelling (Neufeld reaction) with this serotype and observation of agglutination is the usual indication of the reaction of the serotype 37 capsular polysaccharide with the typing sera (P. Landsbo Elverdal, SSI, personal communication).

Electron microscopy of colony lifts (see Figure 1) showed the unusual non-pneumococcal isolates had a distinct cell shape, being less rounded than the pneumococcal cells. There was a visible haze around the very mucoid pneumococcus and there appeared to be a clear area around the cells of the non-mucoid non-pneumococcus serotype 37. The non-encapsulated *S. pseudopneumoniae* type strain (BAA-960$^T$) did not show this clear area.

**Genomic results**

Genomic analysis results using the standard PHE *S. pneumoniae* workflow

Genomic analysis of the panel of 20 study organisms using the standard PHE *S. pneumoniae* bioinformatics workflow (kmerID, MOST and PneumoCaT) are shown in Table 2.
One of the study isolates (PHESPD0357) gave a “best percentage similarity” by kmerID analysis with a *S. pneumoniae* reference, but with a low similarity of 41.0%. The other unusual isolates gave a highest similarity by kmerID (all <40%) with *S. pseudopneumoniae* (either reference strain SK674 or IS7493 uid74453; Table 2). Four of these isolates were from sputum (and had been referred to our laboratory for antibiotic resistance testing due to “unusual resistance” patterns) and three of these isolates had been obtained from blood cultures. (Table 2).

Within this panel, six of the seven unusual clinical isolates gave a similar pattern of unusual results in MLST (all 7 loci giving unrecognised alleles) and kmerID (identity <40% to a *S. pseudopneumoniae* reference). One isolate gave a kmerID match closer to a *S. pneumoniae* reference, but with identity of only 41%. Eleven other clinical isolates with phenotypic characteristics consistent with *S. pneumoniae* gave kmerID matches to the *S. pneumoniae* reference at 77.4-77.9% identity and were all the same MLST type, ST 447. The serotype 37 reference strain SSI-37 possessed a different (but related) MLST profile to the 11 *S. pneumoniae* isolates above and a 77.6% match with the *S. pneumoniae* reference by kmerID. The *S. pseudopneumoniae* type strain BAA-960T gave two unrecognised and five known alleles in the MLST analysis and a match of 99.8% identity to the BAA-960T reference sequence in the kmerID.

The presence of the *tts* gene was detected by mapping of raw WGS reads to a *tts* reference sequence within the PneumoCaT tool. All isolates, apart from BAA-960T, had >98% coverage of the *tts* gene. BAA-960T had no coverage for *tts* (Table 2).
Although type 37 capsule production relies solely on the *tts* gene, *S. pneumoniae* serotype 37 isolates also possess an incomplete 33F-like capsular operon in their genome (Garcia, Llull and López, 1999). The mapping coverage data obtained from the PneumoCaT tool (in which WGS reads from each isolate were mapped to all 92 reference pneumococcal capsular operon sequences as the first stage of the serotype distinction analysis) were used to determine whether the unusual isolates contained regions of known capsular operons. The results are shown in Supplementary Table 3. The results revealed that the seven non-pneumococcal isolates contained DNA sequences that showed coverage for small regions of different capsular operons, but none demonstrated >20% coverage for any of the reference sequences, and the *S. pseudopneumoniae* reference strain BAA-960^T^ showed 26.4% coverage to serotype 36 capsular operon (Supplementary Table 3) and so a pneumococcus-like capsular operon was judged to be absent in these isolates. In comparison, a representative pneumococcal serotype 37 isolate, PHESPV1034, demonstrated >96% coverage of the 33F or 33A capsule operon.

Analysis of the *tts* sequences

Further analysis of the 1530bp *tts* gene sequences in 19 of the 20 study isolates (i.e. excluding BAA-960^T^) revealed that, although there was some nucleotide sequence variation in the study set, NCBI BLAST queries using megablast (optimized for sequences with high-similarity) only returned one hit for all organisms, which was the same *S. pneumoniae* sequence used as the PneumoCaT *tts* reference (GenBank AJ131985.1 positions 2146-3675). BLASTn (optimized for somewhat similar sequences) returned the same sequence as top hit as expected. Translated protein sequences of 509 amino acids could be divided into four main groups. All the
pneumococcal isolates except the SSI-37 matched the translated reference sequence (GenBank AJ131985.1 positions 2146-3675) exactly (see Table 3). The SSI-37 had a unique sequence, with a single amino acid tryptophan -> glycine difference at amino acid position 341 in the protein sequence compared to the 18 others studied (and the reference sequence), which all contained tryptophan at this position. The non-pneumococcal isolates all had the same sequences and only 20 amino acid differences throughout the gene compared to the reference sequence. However, two isolates also contained a DNA insertion in an AT repeat region that led to addition of an extra isoleucine and tyrosine amino acid residue at positions 85 and 86 in the protein sequence for these two isolates.

Analysis of the amino acid substitutions showed that there were five positions in the protein sequence where the non-pneumococcal isolates had amino acid substitutions with negative BLOSUM62 scores (Table 3). This score indicates that this amino acid substitution would be expected to occur only rarely in a homologous protein and may indicate a change in the structure or function of the protein (i.e. the proteins are non-homologous). These substitutions were as follows: position 99 A→D, 247 Y→R, 389 V→F, 473 T→I and 476 T→M. The amino acid difference seen in the SSI-37 reference strain (341 W→G) also had a negative BLOSUM62 score. The properties of the amino acids which could potentially cause functional differences are shown in Supplementary Table 4.

BLASTp search of the protein sequence for each of the groups showed that all had 9/10 top hits with *S. pneumoniae* glycosyltransferase genes.
Evaluation of kmerID %similarity threshold for streptococcal species determination

The results of the threshold analysis intended to inform the selection of a cut-off for the kmerID, are shown in Supplementary Table 5. The results showed that the chosen 65% (representing 0.65 JI value) is a conservative cut-off for a match to *S. pneumoniae* in the kmerID method, which possibly allows for some false-negative classification as non-pneumococcus if an isolate were to demonstrate kmerID similarity of between 60 and 65%.

The neighbour joining tree of all the reference strains used in the kmerID threshold analysis showed *S. pseudopneumoniae* as an out-group to *S. pneumoniae*. *Streptococcus mitis* was most closely related to the pneumococcal/*S. pseudopneumoniae* branch, but with overall low intra-species similarity (Supplementary Figure 1).

Extended kmerID analysis and rMLST

We analysed the genomic data from the isolates in the study panel against an extended streptococcal kmerID database (798 genomes covering 58 streptococcal species) and uploaded assemblies to query the BIGSdb rMLST website for rMLST type and speciation matches. The results of these analyses are shown in Table 4.

Results of the extended kmerID database analysis on the 12 pneumococcal serotype 37 strains (including SSI-37) showed the highest percentage identity (range 80.8 to 83.2%) to the *S. pneumoniae* GA19690 reference genome. Seven *S. pneumoniae* isolates gave the same ribosomal sequence type (rST) 23921. Four *S. pneumoniae* clinical isolates, the SSI-37 type strain and all
of the non-pneumococcal species gave a novel rST. The number of recognized alleles varied
between isolates.

Six of the seven non-pneumococcal isolates were identified only as *Streptococcus* spp. using the
rMLST database speciation tool. Analysis with extended kmerID showed these six gave closest
identity to the *S. mitis* SK1080 genome reference, but as seen with the *S. mitis* reference
sequences the identity was low (range 37-38%).

The remaining non-pneumococcal isolate showed a different identification pattern to the others,
identifying more closely with *S. pneumoniae* by both rMLST speciation (*rpsC*) and both the
original and extended kmerID database, although against the extended kmerID database it
identified closest (41.3% identity) to a different *S. pneumoniae* reference (GA47688) than the
pneumococcal isolates in the study panel.

Whole genome SNP analysis

A maximum likelihood phylogenetic tree of the study set isolates plus extra comparison strains
was constructed. The tree was inferred from the variant alignment derived from SNP variant
analysis using the R6 strain as reference. The analysis involved 36 nucleotide sequences. All
positions with less than 90% site coverage were eliminated. That is, fewer than 10% alignment
gaps, missing data, and ambiguous bases were allowed at any position. There were a total of
63939 positions in the final dataset. The results showed that whole genome SNP analysis also
separated the two groups of *S. pneumoniae* and non-pneumococcal isolates that contain the *tts*
gene (Figure 2). The non-pneumococcal isolates had a common ancestor to the *S. mitis* strains,
but formed a separate branch with strong bootstrap support. Nucleotide differences within and
between the groups of organisms forming the four main branches on the tree (S. pneumoniae, S. pseudopneumoniae, S. mitis and non-pneumococcal tts-positive) showed that the inter-group differences were greater than 5000 SNPs (range 5858 – 7662). The groups showed variable within-group differences, the S. pneumoniae and S. pseudopneumoniae groups showed fewest SNP differences within group (1545 and 3556 respectively) and the S. mitis and the non-pneumococcal tts positive isolates showed the greatest number of within group SNP differences (7811 and 5327 respectively). The distance table is shown in Supplementary Table 6.

Multi-Locus Sequence Analysis

The minimum evolution tree drawn using concatenated MLST allele sequence data for concatenated aroE, gdh, gki, recP, spi, and xpt sequences is shown in Figure 3. As they were all the same MLST type, all the clinical pneumococcal serotype 37 isolates in the study set formed a single branch on the tree with the SSI-37 type strain forming a separate, but closely related branch. The additional S. pneumoniae sequences of various other serotypes added to the analysis formed closely related, but separate branches within the same sub-branch of the tree as the study pneumococcal isolates. The additional S. pseudopneumoniae and S. mitis sequences added to the analysis formed separate clusters on the tree, with the study set of non-pneumococcal sequences forming their own sub-branch near the S. mitis cluster except for the PHESPD0357 isolate, which also produced differing results on initial kmerID and on the rMLST and clustered closer within the S. mitis isolates on the tree rather than on the separate branch.

lytA, ply and piaA gene detection
Detection and analysis of the *lytA*, *ply* and *piaA* genes revealed that all the isolates in the study contained genes with identity to the reference sequences used for *lytA* and *ply*. However, none of the non-pneumococcal isolates contained any sequences homologous to the *piaA* reference sequence or passed the cut-off for detection of *lytA* (80% coverage and 95% identity). Hence, the *piaA* and *lytA* genes were classed as ‘not detected’ in the non-pneumococcal isolates, whereas the *ply* gene was detected in all isolates. The coverage and identity statistics are shown in Table 5.

DISCUSSION

The use of WGS technology in conjunction with bioinformatics analysis will continue to revolutionise reference microbiology and our understanding of bacterial evolution due to the enhanced discrimination of the methodology, and will likely re-write the phylogeny of the bacterial domain. However, how to classify recombinant bacteria with their fluid genomes has been a subject of considerable debate; historically bacteriologists named a species according to common phenotypic traits such as morphology and sugar fermentation (Winslow *et al.*, 1917). In this genomic era, however, it is now commonly discussed if genomic similarity should be the species determinant and whether determining a species is, in fact, possible for fluid bacterial genomes. The Core Genome Hypothesis has been proposed to explain why fluid bacterial genomes can have stable clusters (Riley and Lizotte-Waniewski, 2009) which could be named as species. In this hypothesis it is proposed that core genes are responsible for maintaining the phenotypically distinct species clusters.

In this study, we evaluated both phenotypic and genomic data in an attempt to characterise a number of unusual streptococcal clinical isolates that were discovered in our laboratory using our
standard WGS workflow for *S. pneumoniae*. The workflow includes the kmerID method, MLST and capsular typing, and the combination of these three techniques allowed the recognition of clinically relevant and potentially novel streptococcal isolates that have the *ttt* gene for production of serotype 37 capsular polysaccharide, but which were not *S. pneumoniae*.

SNP analysis and MLST sequence analysis identified these organisms as being similar to, but distinct from isolates identified as *S. pneumoniae* or *S. pseudopneumoniae*, but most closely related to *S. mitis* isolates. As described previously (Hanage et al. 2006) and in our own study, *S. mitis* shows greater genetic diversity than those organisms defined as *S. pneumoniae* or *S. pseudopneumoniae*. Analysis using our kmerID tool to measure whole genome similarity between reference genomes (Supplementary Figure 1) showed that *S. mitis* reference genomes do not co-locate in a distinct branch like other streptococcal species. Instead, individual genomes are separated by long branch lengths. The branch lengths were as long between *S. mitis* reference genomes as those defining the other species (e.g., *S. pneumoniae*, *S. pseudopneumoniae*) in the tree. This was also seen in the analysis of clinical isolates using both whole genome SNP analysis (Figure 2) and MLSA (Figure 3). These data do not support the designation of *S. mitis* as a species, originally defined by serological and biochemical methods (Facklam, 1977). For that reason we can only define the unusual isolates seen in this study as “most similar to *S. mitis*” given that isolates biochemically designated as *S. mitis* are also only “most similar to *S. mitis*” when studied by genomic methods rather than confidently placed within a species branch like *S. pneumoniae* or *S. pseudopneumoniae*. 
Presence of the *tts* gene has not been previously reported in clinical non-pneumococcal isolates to our knowledge. Llull *et al.* (1999) showed that introduction of the *tts* gene to other pneumococcal serotypes and even other Gram-positive organisms *in vitro* caused a binary capsule in pneumococcal isolates containing a capsular operon (García, Llull and López, 1999) and that the *tts* gene alone is responsible for capsular production in organisms without a capsular operon (Llull, Garcia and Lopez, 2001). This was supported by the detection of serotype 37 polysaccharide in five isolates in this study which lacked a capsular operon.

Despite the phylogenetic variation in the group of non-pneumococcal *tts* positive organisms in this study, the TTS protein sequence was identical, therefore it is likely that this gene was transferred by horizontal gene transfer, and evolved and differentiated from that seen in pneumococcus at a point in the past.

Although there were some amino acid substitutions that are only rarely seen in homologous protein sequences according to the BLOSUM62 Matrix, many of these appear to only give slight differences in the amino acid properties. Based on the discussion in Llull *et al.* (Llull, Garcia and Lopez, 2001) the TTS protein contains six transmembrane helices, five of the amino acid substitutions we found in the non-pneumococcal isolates are within these transmembrane helix regions. Only one of these five amino acids had a negative BLOSUM62 score; at position 389, valine was substituted with phenylalanine. These two amino acids differ only by an aromatic ring but share the same hydrophobicity score (0.923 and 0.951 respectively), polarity and lack of H-bond formation, and they both prefer to adopt β-strand conformations.
The difference at residue 247, where tyrosine is present in pneumococcal TTS sequence and arginine in the non-pneumococcal sequence, is the change with the most impact on amino acid properties, from a relatively hydrophobic amino acid in the pneumococcal protein to a very hydrophilic one.

It is unlikely that these differences in sequence would dramatically change the function of the TTS protein compared to that found in *S. pneumoniae*, but this could only be confirmed by full structural and functional analysis.

The TTS amino acid sequence appears to be stable and it therefore not a recent acquisition by one particular clone of these organisms. There is no evidence of clonal expansion of these *tts* positive organisms in the isolates we have found.

Further investigation is needed to determine the regulation of the *tts* gene expression and polysaccharide production in the organisms characterised in this study, as there was clearly a difference between the capsular expression for the *S. pneumoniae* serotype 37 isolates in which the capsule is abundant and mucoid, and the unusual isolates which appear to express far less polysaccharide and have a non-mucoid appearance. It is possible some parts of the redundant regular capsular operon or other external factors may play some part in the augmentation of expression of the phenotype in the pneumococcus, and certainly these factors warrant further study. Non-encapsulated pneumococci and non-pneumococcal streptococci play a major role in horizontal transfer of genetic information, their acapsular phenotype allowing more ready interchange of genetic information than capsulated organisms (Chewapreecha *et al.*, 2014). The
seemingly limited expression of the polysaccharide in these \textit{tts} positive non-pneumococcal organisms could mean transfer of genetic information may be more likely than in the pneumococcus, leading to this version of the \textit{tts} gene being found in more diverse organisms. These isolates appear to be rare, mostly non-invasive and may not normally be identified as \textit{S. pneumoniae} on the bench of a routine microbiology laboratory. Therefore, typically they would not be referred for serotyping to a reference laboratory such as the PHE \textit{S. pneumoniae} reference laboratory for serotyping and thus they would remain unidentified. Even if referred to reference laboratories such isolates may also be misidentified as pneumococcal serotype 37 or non-typeable organisms unless WGS methods are used.

In the future, thorough WGS analyses for pneumococcal serotype, which include analysis of the entire capsular operon and necessary related genes (such as \textit{tts}), should be more sensitive to the detection of potentially novel/mixed results (e.g., binary capsules, should they exist \textit{in vivo}), than methods that focus on smaller regions of the sequence. A recent study of capsular production in non-pneumococcal species such as \textit{S. mitis} and \textit{S. oralis} showed that the production of capsule via the wzx-wzy pathway in these species is not uncommon and some bear antigenic and genetic similarity to those recognised for \textit{S. pneumoniae} which could lead to misidentification of these species (Skov Sørensen \textit{et al.}, 2016). Previous studies have also shown an abundance of pneumococcal capsular gene homologs present in non-pneumococcal streptococci which can confound PCR based serotyping methods (Carvalho \textit{et al.}, 2013). However, we have not yet found any examples of misidentified isolates of this nature in the clinical isolates we have studied to date using our WGS methods which should, as seen with the
serotype 37 isolates, be sensitive enough to recognise any previously misidentified isolates by combined detection of capsular operon and use of whole genome kmer analysis for species identification.

For laboratory identification of these isolates, which could be potentially misidentified as pneumococcal serotype 37, DNA sequence-based methods were superior to phenotypic methods for obtaining a clear differentiation between *S. pneumoniae* and closely related species. Although *lytA* was defined as ‘not-detected’ in the non-pneumococcal isolates using our coverage and identity cut-off, the presence of both *lytA*-like and *ply* sequences in all of these strains may confound species identification methods based on short-sequence detection such as PCR or oligonucleotide probe hybridization, however the alternative target, *piaA*, which has previously been suggested as a specific pneumococcal PCR target (Whalan et al. 2006; Trzciński et al. 2013) does appear to offer species differentiation in these organisms.

Currently the standard *S. pneumoniae* seven allele MLST scheme offers a suitable alternative to WGS for determining whether an isolate is a pneumococcus or not. In our study, the presence of multiple unrecognised alleles (>1) in the standard pneumococcal MLST scheme (PubMLST database accessed October 2016) correlated with the WGS kmerID identification as non-pneumococcal species. When the whole genome analysis gave a result of <65% identity to *S. pneumoniae* by kmerID, the MLST also gave multiple unrecognised alleles (>1 locus). This was also supported by the use of the concatenated sequence data from six of the alleles in the MLSA analysis (excluding *ddl*) which was useful for producing phylogenetic trees and may also be used to help assign an identity if comparative species sequences are available. If the presence of
multiple novel alleles in the standard MLST data is to be used as an indication of a non-
pneumococcal species, it is important that the database of MLST alleles for *S. pneumoniae*,
hosted at PubMLST, is kept free of sequences from isolates misidentified as pneumococcus. Up
to the time of our study, well-managed curation of the *S. pneumoniae* MLST database appears to
have excluded allele sequences that show excessive variation from standard *S. pneumoniae*
sequences, thus suggesting non-pneumococcal origin, and has so far enabled the database to be
useful in determining whether an isolate is a pneumococcus or not simply by the number of
novel alleles seen in the organisms.

Limited clinical information was available for the non-pneumococcal *tts* positive isolates. Of the
three isolates that were obtained from blood cultures, two of the patients had pneumonia and one
bacteraemia. Two of the isolates from blood cultures came from patients with co-morbidities
(immunosuppression in one and congenital mitochondrial cytopathy in another), suggesting that
although they can be seen in invasive disease these organisms may also be opportunistic and
require more susceptible hosts to enable invasion to normally sterile sites. Clinical details are
shown in Supplementary Table 7.

The abundance of these non-pneumococcal species containing the *tts* gene and their potential
clinical relevance compared to pneumococcus is unknown and they are likely to be often
misidentified in the routine laboratory. However, the increasing use of molecular and, in
particular, whole genome analysis techniques in reference laboratories should increase the
likelihood of identification of these organisms. This will allow further opportunities to study
their molecular and clinical characteristics and enable the further description or classification of
fluid bacterial species like those in the S. mitis group.

ACKNOWLEDGEMENTS
The authors would like to thank our colleagues at PHE, London UK, including McDonald Prest
for retrieving isolates from storage, DNA extraction and performing bile solubility tests, Ella
Campion, Gurkiran Mankoo, Sophie Hang, and John Duncan for serotyping isolates, Richard
Myers, for discussion on kmerID and phylogenetics and Sarah Collins of the Immunisation
Department for providing matched clinical information.

We would like to thank Pernille Landsbo Elverdal (SSI Diagnostica, Denmark), for discussions
on serotyping of 37 isolates.

We would also like to thank Bill Hanage (Harvard University, USA) who participated in helpful
discussions on MLSA.

REFERENCES
Balsalobre, L., Hernández-Madrid, A., Llull, D., Martín-Galiano, A. J., García, E., Fenoll, A., de
la Campa, A. G., Hernandez-Madrid, A., Llull, D., Martin-Galiano, A. J., Garcia, E., Fenoll, A.
and de la Campa, A. G. (2006) ‘Molecular characterization of disease-associated streptococci of
the mitis group that are optochin susceptible’, Journal of Clinical Microbiology. American
Society for Microbiology, 44(11), pp. 4163–4171. doi: 10.1128/JCM.01137-06.

Bentley, S. D., Aanensen, D. M., Mavroidi, A., Saunders, D., Rabbinowitsch, E., Collins, M., Donohoe, K., Harris, D., Murphy, L., Quail, M. A., Samuel, G., Skovsted, I. C., Kaltoft, M. S., Barrell, B., Reeves, P. R., Parkhill, J. and Spratt, B. G. (2006) ‘Genetic Analysis of the Capsular Biosynthetic Locus from All 90 Pneumococcal Serotypes’, PLoS Genetics. Public Library of Science, 2(3), p. e31. doi: 10.1371/journal.pgen.0020031.

Carvalho, M. da G., Pimenta, F. C., Moura, I., Roundtree, A., Gertz, R. E., Li, Z., Jagero, G., Bigogo, G., Junghae, M., Conklin, L., Feikin, D. R., Breiman, R. F., Whitney, C. G. and Beall, B. W. (2013) ‘Non-pneumococcal mitis-group streptococci confound detection of pneumococcal capsular serotype-specific loci in upper respiratory tract.’, PeerJ, 1(1), p. e97. doi: 10.7717/peerj.97.

Chewapreecha, C., Harris, S. R., Croucher, N. J., Turner, C., Marttinen, P., Cheng, L., Pessia, A., Aanensen, D. M., Mather, A. E., Page, A. J., Salter, S. J., Harris, D., Nosten, F., Goldblatt, D., Corander, J., Parkhill, J., Turner, P. and Bentley, S. D. (2014) ‘Dense genomic sampling identifies highways of pneumococcal recombination.’, Nature genetics, 46(3), pp. 305–9. doi: 10.1038/ng.2895.

Facklam, R. R. (1977) ‘Physiological differentiation of viridans streptococci.’, Journal of clinical microbiology, 5(2), pp. 184–201.

García, E., Llull, D. and López, R. (1999) ‘Functional organization of the gene cluster involved in the synthesis of the pneumococcal capsule.’, International microbiology : the official journal of the Spanish Society for Microbiology, 2(3), pp. 169–176.
Glaeser, S. P. and Kämpfer, P. (2015) ‘Multilocus sequence analysis (MLSA) in prokaryotic taxonomy’, Systematic and Applied Microbiology, 38(4), pp. 237–245. doi: 10.1016/j.syapm.2015.03.007.

Hanage, W. P., Fraser, C. and Spratt, B. G. (2006) ‘Sequences, sequence clusters and bacterial species’, Philos Trans R Soc Lond B Biol Sci, 361(1475), pp. 1917–1927. doi: 10.1098/rstb.2006.1917.

Henikoff, S. and Henikoff, J. G. (1992) ‘Amino acid substitution matrices from protein blocks.’, Proceedings of the National Academy of Sciences of the United States of America, 89(22), pp. 10915–9.

Johnston, C., Hinds, J., Smith, A., van der Linden, M., Van Eldere, J. and Mitchell, T. J. (2010) ‘Detection of large numbers of pneumococcal virulence genes in streptococci of the mitis group’, Journal of Clinical Microbiology. American Society for Microbiology, 48(8), pp. 2762–2769. doi: 10.1128/JCM.01746-09.

Jolley, K. A., Bliss, C. M., Bennett, J. S., Bratcher, H. B., Brehony, C., Colles, F. M.,
Wimalaratna, H., Harrison, O. B., Sheppard, S. K., Cody, A. J. and Maiden, M. C. J. (2012) ‘Ribosomal multilocus sequence typing: Universal characterization of bacteria from domain to strain’, Microbiology, 158(4), pp. 1005–1015. doi: 10.1099/mic.0.055459-0.

Kapatai, G., Sheppard, C. L., Al-Shahib, A., Litt, D. J., Underwood, A. P., Harrison, T. G. and Fry, N. K. (2016) ‘Whole genome sequencing of Streptococcus pneumoniae : development, evaluation and verification of targets for serogroup and serotype prediction using an automated pipeline’, PeerJ. PeerJ Inc., 4, p. e2477. doi: 10.7717/peerj.2477.
Keith, E. R., Podmore, R. G., Anderson, T. P. and Murdoch, D. R. (2006) ‘Characteristics of Streptococcus pseudopneumoniae isolated from purulent sputum samples’, *Journal of Clinical Microbiology*, 44(3), pp. 923–927. doi: 10.1128/JCM.44.3.923-927.2006.

Kumar, S., Stecher, G. and Tamura, K. (2016) ‘MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets.’, *Molecular biology and evolution*, p. msw054. doi: 10.1093/molbev/msw054.

Langmead, B. (2010) ‘Aligning short sequencing reads with Bowtie.’, *Current protocols in bioinformatics / editorial board, Andreas D. Baxevanis ... [et al.].* NIH Public Access, Chapter 11, p. Unit 11.7. doi: 10.1002/0471250953.bi1107s32.

Li, H. and Durbin, R. (2009) ‘Fast and accurate short read alignment with Burrows-Wheeler transform.’, *Bioinformatics (Oxford, England).* Oxford University Press, 25(14), pp. 1754–60. doi: 10.1093/bioinformatics/btp324.

Llull, D., Garcia, E. and Lopez, R. (2001) ‘Tts, a processive beta-glucosyltransferase of Streptococcus pneumoniae, directs the synthesis of the branched type 37 capsular polysaccharide in Pneumococcus and other gram-positive species.’, *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 276(24), pp. 21053–61. doi: 10.1074/jbc.M010287200.

Llull, D., Muñoz, R., López, R. and García, E. (1999) ‘A Single Gene ( tts ) Located outside the cap Locus Directs the Formation of *Streptococcus pneumoniae* Type 37 Capsular Polysaccharide’, *The Journal of Experimental Medicine*. Rockefeller Univ Press, 190(2), pp. 241–252. doi: 10.1084/jem.190.2.241.
McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M. and DePristo, M. A. (2010) ‘The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data.’, 

*Genome research.* Cold Spring Harbor Laboratory Press, 20(9), pp. 1297–303. doi: 10.1101/gr.107524.110.

Nelson, A. L., Roche, A. M., Gould, J. M., Chim, K., Ratner, A. J. and Weiser, J. N. (2007) ‘Capsule Enhances Pneumococcal Colonization by Limiting Mucus-Mediated Clearance’, 

*Infection and Immunity.* American Society for Microbiology, 75(1), pp. 83–90. doi: 10.1128/IAI.01475-06.

Riley, M. A. and Lizotte-Waniewski, M. (2009) ‘Population genomics and the bacterial species concept.’, *Methods in molecular biology (Clifton, N.J).* NIH Public Access, 532, pp. 367–77. doi: 10.1007/978-1-60327-853-9_21.

Rzhetsky, A. and Nei, M. (1992) ‘A simple method for estimating and testing minimum evolution trees.’, *Molecular Biology and Evolution*, 9, pp. 945–967.

Skov Sørensen, U. B., Yao, K., Yang, Y., Tettelin, H. and Kilian, M. (2016) ‘Capsular Polysaccharide Expression in Commensal Streptococcus Species: Genetic and Antigenic Similarities to Streptococcus pneumoniae.’, *mBio.* American Society for Microbiology, 7(6), pp. e01844-16. doi: 10.1128/mBio.01844-16.

Stamatakis, A. (2014) ‘RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies.’, *Bioinformatics (Oxford, England).* Oxford University Press, 30(9), pp. 1312–3. doi: 10.1093/bioinformatics/btu033.
Tewolde, R., Dallman, T., Schaefer, U., Sheppard, C. L., Ashton, P., Pichon, B., Ellington, M., Swift, C., Green, J. and Underwood, A. (2016) ‘MOST: a modified MLST typing tool based on short read sequencing’, PeerJ. PeerJ Inc., 4, p. e2308. doi: 10.7717/peerj.2308.

Waight, P. A., Andrews, N. J., Ladhani, S. N., Sheppard, C. L., Slack, M. P. E. and Miller, E. (2015) ‘Effect of the 13-valent pneumococcal conjugate vaccine on invasive pneumococcal disease in England and Wales 4 years after its introduction: an observational cohort study’, The Lancet Infectious Diseases, 15(5), pp. 535–543. doi: 10.1016/S1473-3099(15)70044-7.

Whatmore, A. M., Efstratiou, A., Pickerill, A. P., Broughton, K., Woodard, G., Sturgeon, D., George, R. and Dowson, C. G. (2000) ‘Genetic relationships between clinical isolates of Streptococcus pneumoniae, Streptococcus oralis, and Streptococcus mitis: Characterization of “atypical” pneumococci and organisms allied to S. mitis harboring S. pneumoniae virulence factor-encoding genes’, Infection and Immunity, 68(3), pp. 1374–1382. doi: 10.1128/IAI.68.3.1374-1382.2000.

Winslow, C. E., Broadhurst, J., Buchanan, R. E., Krumwiede, C., Rogers, L. A., Smith, G. H. and Smith, G. H. (1917) ‘The Families and Genera of the Bacteria: Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types.’, Journal of bacteriology. American Society for Microbiology (ASM), 2(5), pp. 505–66.

Yother, J. (2011) ‘Capsules of Streptococcus pneumoniae and Other Bacteria: Paradigms for Polysaccharide Biosynthesis and Regulation’, Annual review of microbiology. Annual Reviews, 65, pp. 563–81. doi: 10.1146/annurev.micro.62.081307.162944.
Table 1 (on next page)

Phenotypic analysis of serotype 37 isolates, with BAA-960\(^T\) as a *Streptococcus pseudopneumoniae* reference.
Table 1. Phenotypic analysis of serotype 37 isolates, with BAA-960\textsuperscript{T} as a *Streptococcus pseudopneumoniae* reference.

| Isolate     | Colony appearance | Serotype by slide agglutination | Optochin sensitivity | Bile solubility | API 32 Strep profile | API species identification | API % |
|-------------|-------------------|---------------------------------|----------------------|-----------------|----------------------|---------------------------|-------|
| PHESPD0338  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 72076741110          | *S. pneumoniae*            | 88.0  |
| PHESPD0344  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 40272741110          | *S. pneumoniae*            | 99.9  |
| PHESPD0356  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 46072741100          | *S. pneumoniae*            | 77.5  |
| PHESPD0383  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 60076741110          | *S. pneumoniae*            | 99.9  |
| PHESPV0691  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 76076741110          | *S. pneumoniae*            | 87.4  |
| PHESPV1034  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 40076741100          | *S. pneumoniae*            | 99.6  |
| PHESPV1405  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 60076741110          | *S. pneumoniae*            | 99.9  |
| PHESPD0363  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 46072741100          | *S. pneumoniae*            | 77.5  |
| PHESPV0789  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 40072601100          | *S. pneumoniae*            | 97.9  |
| PHESPV1119  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 50076741100          | *S. pneumoniae*            | 99.9  |
| PHESPD0355  | Very Mucoid       | 37                              | Sensitive            | Soluble         | 50072541100          | *S. pneumoniae*            | 99.9  |
| SSI-37      | Very Mucoid       | 37                              | Sensitive            | Soluble         | 52076741100          | *S. pneumoniae*            | 98.3  |
| PHESPD0357  | Small             | 37                              | Sensitive            | Insoluble       | 00132541100          | *S. mitis*                 | 97.3  |
| PHENP00003  | Small             | 37                              | Sensitive            | Indeterminate   | 40036441100          | *S. oralis*                | 95.3  |
| PHENP00005  | Small             | 37                              | Sensitive            | Indeterminate   | 40016441100          | *S. oralis*                | 90.6  |
| PHENP00006  | Small             | 37                              | Sensitive            | Indeterminate   | 42052541100          | *S. pneumoniae*            | 92.9  |
| PHENP00001  | Very Small        | 37                              | Sensitive            | Indeterminate   | 40116441120          | *S. oralis*                | 94.6  |
| PHENP00002  | Very Small        | Untypeable                      | Sensitive            | Indeterminate   | 40112441110          | *S. mitis*                 | 95.2  |
| PHENP00007  | Very Small        | Untypeable                      | Sensitive            | Indeterminate   | 60016441100          | *S. oralis*                | 96.0  |
| BAA-960\textsuperscript{T} | Rough       | Not done                        | Sensitive            | Indeterminate   | 64012441140          | *S. oralis*                | 98.3  |
Table 2 (on next page)

Serotype 37 (tts gene detected) isolates with standard *Streptococcus pneumoniae* WGS workflow analysis results, including the *S. pseudopneumoniae* (BAA-960<sup>T</sup>) and SSI serotype 37 strains for reference.

CSF, cerebrospinal fluid; * = novel allele
Table 2. Serotype 37 (*tt* gene detected) isolates with standard *Streptococcus pneumoniae* WGS workflow analysis results, including the *S. pseudopneumoniae* (BAA-960) and SSI serotype 37 strains for reference.

| Isolate number | Isolation site | MLST Sequence type | MLST profile (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, *ddl*) | *tt* gene | kmerID top match reference genome | kmerID % similarity |
|----------------|----------------|--------------------|---------------------------------------------------------------|----------|-----------------------------------|---------------------|
| PHESPD0338     | Blood          | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.4                |
| PHESPD0344     | Blood          | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.4                |
| PHESPD0356     | Blood          | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.3                |
| PHESPD0383     | Blood          | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.3                |
| PHESPV0691     | CSF            | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.4                |
| PHESPV1034     | Blood          | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 79.7                |
| PHESPV1405     | Blood          | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.5                |
| PHESPD0363     | Blood          | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.7                |
| PHESPV0789     | Unknown        | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.9                |
| PHESPV1119     | Blood          | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.5                |
| PHESPV0355     | Unknown        | 447                | 29, 33, 19, 1, 36, 22, 31                                     | +        | *Streptococcus pneumoniae* 5652-06 | 77.4                |
| SSI-37         | Unknown        | 7243               | 192, 34, 19, 1, 36, 22, 445                                  | +        | *Streptococcus pneumoniae* 5652-06 | 77.6                |
| PHESPD0357     | Blood          | Novel              | all loci novel                                               | +        | *Streptococcus pseudopneumoniae* | 41.0                |
| PHENP00003     | Sputum         | Novel              | all loci novel                                               | +        | *Streptococcus pseudopneumoniae* IS7493 uid71153 | 34.6 |
| PHENP00005     | Sputum         | Novel              | all loci novel                                               | +        | *Streptococcus pseudopneumoniae* IS7493 uid71153 | 37.0 |
| PHENP00006     | Sputum         | Novel              | all loci novel                                               | +        | *Streptococcus pseudopneumoniae* IS7493 uid71153 | 36.4 |
| PHENP00001     | Sputum         | Novel              | all loci novel                                               | +        | *Streptococcus pseudopneumoniae* SK674 | 33.8 |
| PHENP00002     | Blood          | Novel              | all loci novel                                               | +        | *Streptococcus pseudopneumoniae* SK674 | 33.8 |
| PHENP00007     | Blood          | Novel              | all loci novel                                               | +        | *Streptococcus pseudopneumoniae* SK674 | 34.0 |
| BAA-960        | Unknown        | Novel              | 139, 371, 345, *, 441, *, 656                                | -        | *Streptococcus pseudopneumoniae* IS7493 uid71153 | 99.8 |

3 CSF, cerebrospinal fluid; * = novel allele
Table 3 (on next page)

Sites within the TTS sequence that show amino acid variability for 19 study panel isolates (with amino acid codes), including BLOSUM62 score for likelihood of substitution in a homologous protein.

* Amino acids insertions. All other stated amino acid positions are referenced according to the sequence in isolates that do not contain the insertions; n/a. not applicable

+ Residues in with positive scores (green) substitute frequently in homologous proteins (positive BLOSUM62 score), while those in red substitute rarely.
| Isolate    | Amino acid position |
|------------|---------------------|
|            | 17 | 79 | 82 | 85 | 86 | 88 | 89 | 99 | 11 | 12 | 24 | 24 | 34 | 34 | 37 | 38 | 41 | 46 | 47 | 47 | 47 | 49 | 50 |
| tts reference |    |    |    | *  | *  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| PHESPD033 8 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPD034 4 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPD035 6 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPD038 3 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPV069 1 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPV103 4 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPV140 5 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPD036 3 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPV078 9 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPV111 9 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |
| PHESPD035 5 | S  | N  | H  | -  | -  | S  | S  | A  | V  | A  | K  | Y  | W  | V  | F  | V  | V  | L  | T  | T  | I  | A  | V  |    |

**Table 3.** Sites within the TTS protein sequence that show amino acid variability for 19 study panel isolates (with amino acid codes), including BLOSUM62 score for likelihood of substitution in a homologous protein.
|   | PHESPD035  | N N D A V N R W I Y F L F I M F V L |
|---|------------|-----------------------------------|
| 7 | T K Y - - | N N D A V N R W I Y F L F I M F V L |
| PHENP0000 3 | T K Y - - | N N D A V N R W I Y F L F I M F V L |
| PHENP0000 5 | T K Y - - | N N D A V N R W I Y F L F I M F V L |
| PHENP0000 6 | T K Y - - | N N D A V N R W I Y F L F I M F V L |
| PHENP0000 7 | T K Y - - | N N D A V N R W I Y F L F I M F V L |
| PHENP0000 1 | T K Y I Y N N D A V N R W I Y F L F I M F V L |
| PHENP0000 2 | T K Y I Y N N D A V N R W I Y F L F I M F V L |
| BLOSUM62 Score + | 1 0 2 n/a | 1 -2 0 0 0 -2 2 1 0 -1 0 0 1 |

* amino acids insertions. All other stated amino acid positions are referenced according to the sequence in isolates that do not contain the insertions; n/a not applicable.

+ Residues in with positive scores (green) substitute frequently in homologous proteins (positive BLOSUM62 score), while those in red substitute rarely.
Table 4 (on next page)

Further genomic analysis, extended kmerID database and rMLST.

- = no match, ND = Not Done
Table 4. Further genomic analysis, extended kmerID database and rMLST.

| Isolate     | PubMLST BIGSdb rMLST | Speciation match (single rps allele) | Speciation first match gene | top match reference genome | Similarity % |
|-------------|----------------------|--------------------------------------|-----------------------------|---------------------------|--------------|
|             | rST                  | profile match species                | No. Alleles matched         |                           |              |
| PHESPD033   | 2392 1               | S. pneumoniae                        | 52                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 81.1         |
| PHESPD034   | 2392 1               | S. pneumoniae                        | 52                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 81.1         |
| PHESPD035   | 2392 1               | S. pneumoniae                        | 52                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 80.8         |
| PHESPD038   | 2392 1               | S. pneumoniae                        | 52                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 80.9         |
| PHESPV069   | 2392 1               | S. pneumoniae                        | 52                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 81.1         |
| PHESPV103   | 2392 1               | S. pneumoniae                        | 52                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 83.2         |
| PHESPV140   | 2392 1               | S. pneumoniae                        | 52                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 81.1         |
| PHESPD036   | novel                | –                                    | 51                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 81.2         |
| PHESPV078   | novel                | –                                    | 51                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 81.1         |
| PHESPV111   | novel                | –                                    | 50                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 81.1         |
| PHESPD035   | novel                | –                                    | 51                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 81.1         |
| SSI-37      | novel                | –                                    | 48                          | S. pneumoniae rpsA        | S. pneumoniae GA19690 | 81.3         |
| Sample ID   | GenBank Accession | Organism(s) | Gene | Reference Organism | Reference GenBank Accession | Identity (%) |
|------------|------------------|-------------|------|--------------------|----------------------------|--------------|
| PHESPD035  | novel            | –           | 37   | S. pneumoniae      | rpsC                       | S. pneumoniae GA19690 | 41.3         |
| PHENP0000  | novel            | –           | 47   | Streptococcus spp. | rpsB                       | S. mitis SK1080     | 36.9         |
| PHENP0000  | novel            | –           | 39   | Streptococcus spp. | rpsB                       | S. mitis SK1080     | 37.1         |
| PHENP0000  | novel            | –           | 40   | Streptococcus spp. | rpsB                       | S. mitis SK1080     | 38.0         |
| PHENP0000  | novel            | –           | 48   | Streptococcus spp. | rpsA                       | S. mitis SK1080     | 37.1         |
| PHENP0000  | novel            | –           | 48   | Streptococcus spp. | rpsA                       | S. mitis SK1080     | 37.4         |
| PHENP0000  | novel            | –           | 52   | Streptococcus spp. | rpsA                       | S. mitis SK1080     | 37.5         |
| BAA-960^T  | 3104T            | S. pseudopneumoniae | 52   | S. pseudopneumoniae | rpsA                       | ND            | ND           |

- = no match, ND = Not Done
Table 5 (on next page)

lytA, ply and piaA gene detection, coverage and identity statistics for isolates in the study.
Table 5. *lytA*, *ply* and *piaA* gene detection, coverage and identity statistics for isolates in the study.

| Isolate         | lytA coverage % | lytA % identity to KP110770 | ply coverage % | ply identity to HG531769 | piaA coverage % | piaA identity to AF338658.1:111-1130 |
|-----------------|-----------------|------------------------------|----------------|---------------------------|-----------------|--------------------------------------|
| PHESPD0338      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPD0344      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPD0356      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPD0383      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPV0691      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPV1034      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPV1405      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPD0363      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPV0789      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPV1119      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPD0355      | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| SSI-37          | 100             | 99.58                        | 100            | 99.65                     | 100             | 99.6                                 |
| PHESPD0357      | 88              | 73.25                        | 100            | 96.96                     | 0               | 0                                    |
| PHENP00003      | 85.8            | 76.28                        | 100            | 97.1                      | 0               | 0                                    |
| PHENP00005      | 92.7            | 78.16                        | 100            | 95.41                     | 0               | 0                                    |
| PHENP00006      | 93.3            | 77.32                        | 100            | 95.34                     | 0               | 0                                    |
| PHENP00001      | 78.2            | 67.92                        | 100            | 97.03                     | 0               | 0                                    |
| PHENP00002      | 86.3            | 75.03                        | 100            | 96.75                     | 0               | 0                                    |
| PHENP00007      | 87.4            | 75.44                        | 100            | 96.19                     | 0               | 0                                    |
| BAA-960T        | 91.8            | 78.47                        | 100            | 96.68                     | 0               | 0                                    |
Figure 1

Electron micrographs of A: mucoid serotype 37 pneumococcus PHESPV1034, B: unusual serotype 37 PHENP00003, C: BAA-960 Streptococcus pseudopneumoniae. Scale bar 1um.
Figure 2

Whole genome SNP analysis maximum likelihood tree (RAxML bipartition) of study set isolates using the acapsular R6 reference.

Blue squares represent study set of *S. pneumoniae* isolates, purple squares the *S. pseudopneumoniae* type strain and red squares the study set non-pneumococcal isolates. Additional contextual data were provided by *Streptococcus pneumoniae* belonging to different serotypes (N=5, blue circles), *S. pseudopneumoniae* (N=6, purple circles) and *S. mitis* strains (N=5, green circles).

Branch length corresponds to substitutions per base. Bootstrap support shown for all branches.
Figure 3

Minimum evolution tree of concatenated MLST sequences (except *ddl*) of study set isolates with contextual isolates.

Blue squares represent *S. pneumoniae* isolates, purple squares the *S. pseudopneumoniae* type strain and red squares non-pneumococcal isolates included in this study. Additional contextual data from other *Streptococcus pneumoniae* serotypes (blue circles), *S. pseudopneumoniae* (purple circles) and *S. mitis* strains (green circles).