Defining the Paediatric Nasopharyngeal Microbiota during PCV13 Vaccine Implementation

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ABSTRACT

During pneumococcal conjugate vaccine (PCV) implementation, studies have shown a reduction in the carriage of serotypes included within the vaccine and differing reports of the indirect effects this reduction has had on other species of the nasopharynx. This study contributes to and expands upon this area of work, focusing on PCV13 implementation, which occurred four years after the introduction of PCV7, and its effects on pneumococcal carriage and other species carried in the upper respiratory tract.

Between 2006/07 and 2012/13, PCV13 vaccine serotypes reduced in carriage with a corresponding increase in PCV13 non-vaccine serotypes, and an overall consistent rate of pneumococcal carriage. No change in carriage rate was detected with any of the bacterial species detected at the species level. Carriage of *H. influenzae* was determined to be predominately non-typeable *H. influenzae* (NTHi) and demonstrated clonal diversity when typed using MLST. Antibiotic resistance genes were detected in around 10% of pneumococcal and *H. influenzae* isolates, with *ermB* and blaTEM genes being most prevalent for each species respectively. Virulence factor profiling resulted in matching gene profiles being ST dependent for *S. pneumoniae* and *H. influenzae*. Pneumococcal serotype 15A/ST63 has emerged as being a prevalent strain post-PCV13 with the propensity to contribute to both antibiotic resistance and virulence.

This thesis provides information relevant to vaccine policy based on carriage data and expands upon the neglected area of using next generation sequencing and molecular characterisation of carriage isolates.

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"All that I am, or ever hope to be, I owe to my angel Mother."

Abraham Lincoln

ABBREVIATIONS

AHS – Alpha haemolytic streptococci (not including *S. pneumoniae* spp)

BlaTEM – Beta-lactamase (gene)

CI – 95% confidence interval

HI – *Haemophilus influenzae*

HIB – *Haemophilus influenzae* B (vaccine)

IPD – Invasive pneumococcal disease

MC – *Moraxella catarrhalis*

MLST – Multi locus sequence typing

MRSA – Meticillin resistant *Staphylococcus aureus*

MSSA – Meticillin sensitive *Staphylococcus aureus*

NIP – National immunisation program

NM – *Neisseria meningitidis*

NTHi – Non-typeable *Haemophilus influenzae*

PCR – Polymerase chain reaction

PCV – Pneumococcal conjugate vaccine

PPS – Plain polysaccharide (vaccine)

SPN – *Streptococcus pneumoniae*

1. INTRODUCTION

1.1. THE BURDEN OF RESPIRATORY INFECTION

1.1.1. RESPIRATORY TRACT INFECTIONS

According to the World Health Organization's burden of disease report, lower respiratory infections are the third biggest cause of death in the world causing 6.1% of all deaths (The World Health Organisation, 2011). Not only is there a serious health risk associated with these conditions, they are also costly to health systems such as the UK National Health Service (NHS). In 2008, antibiotic prescribing for respiratory infections alone was estimated to cost the UK government £24 million (National Institute for Health and Clinical Excellence, 2008). Common infections of the upper respiratory tract (URT) include rhinitis, rhinosinusitis, nasopharyngitis, laryngitis and otitis media (OM), whilst infections of the lower respiratory tract (LRT) include emphysema, pneumonia, bronchitis and tuberculosis. Common causative agents include bacteria and viruses; from these LRT infections *Streptococcus pneumonia*e and *Haemophilus influenzae* are commonly cultured (Tenenbaum et al., 2012).

1.1.2. NON-INVASIVE AND INVASIVE DISEASE

Pneumococcal disease can be divided broadly into two groups, invasive pneumococcal disease (IPD) and non-invasive pneumococcal disease (non-IPD). IPD is defined as dissemination of pneumococcal bacteria from the initial site of colonisation to normally sterile sites; examples are sepsis, bacteraemia, pneumococcal meningitis and bacteraemic pneumonia (Avni et al., 2010, Public Health England, 2014a). Sepsis occurs as a whole body inflammatory response to an

infectious organism, such as *S. pneumoniae*, when present in the blood or tissues of a host, and which may be fatal. Bacterial meningitis is an inflammation of the meninges due to a bacterial infection. Non-invasive pneumococcal diseases include non-bacteraemic pneumonia, middle ear infection (otitis media), sinusitis, and bronchitis. Risk-groups will be affected to a greater extent, particularly the young, the elderly, the immuno-compromised and those with a long-term health condition (Public Health England, 2013). Pneumonia is currently considered non-invasive if bacteria are cultured only from respiratory specimens but may be invasive if pneumococci are able to infiltrate the blood stream (Ortqvist et al., 2005). To gain a true understanding of the burden of pneumonia, it is important to monitor not only 'classic' invasive bacteraemic pneumonia but also non-bacteraemic pneumonia infections as it has been estimated that there are three non-invasive infections for each invasive infection (Feldman and Anderson, 2014, Said et al., 2013).

IPD is a cause of substantial morbidity and mortality world-wide. In 2000, there were 735,000 pneumococcal deaths (The World Health Organisation, 2012) (Table 1.1), of these deaths, 61% were associated with just ten countries in Asia and Africa, India (27%), China (12%), Nigeria (5%), Pakistan (5%), Bangladesh (4%), Indonesia (3%), Ethiopia (3%), Congo (3%), Kenya (2%), and the Philippines (2%), demonstrating pneumococcal disease to be a substantial cause of mortality in developing countries (O'Brien et al., 2009). In the UK, up to 6,000 cases and an additional 40,000 hospitalisations due to pneumococcal pneumonia are reported per year (Health Protection Agency, 2010).

H. influenzae is associated with similar disease manifestations, (invasive and non-invasive infections), to those described for *S. pneumoniae*. Prior to 1992, *H. influenzae* b (Hib) was the single largest cause of bacterial meningitis worldwide. In the US, it was associated with severe health implications for ~20% of survivors and a 4% fatality rate (Elizabeth C. Briere, 2011, Broome, 1987). In the UK, between 1985

and 1991, it has been estimated that there were 900 cases per year of Hib meningitis of which 60 cases ended in fatality (Heath and McVernon, 2002, Booy et al., 1993). Between 2004 and 2011 there was a total incidence of 133 reported cases of meningitis caused by any strain of *H. influenzae* in the UK population (Okike et al., 2014), however the WHO estimates that in 2008 there were 199,000 deaths in children younger than five years of age worldwide due to Hib (The World Health Organisation, 2012) (Table 1.1).

Infants who are too young to have received the Hib vaccination remain at risk of infection if Hib circulates in the general population (Leung et al., 2012). Recent studies have concluded that NTHi and the other serotypes of *H. influenzae* have demonstrated capability to cause invasive disease (Kilic et al., 2010, van Wessel et al., 2011, Ladhani et al., 2012), thus surveillance of all *H. influenzae* strains is of importance. Information gained from such surveillance can subsequently inform vaccine policy, as new preventative methods may be needed for other serotypes and unencapsulated strains. It is currently unknown whether the increase of reported cases of non-Hib invasive disease, caused by either NTHi or non-Hib serotypes, is due to serotype replacement or if it is due to advances in detection methods. A 'true' change would imply that these previously non-invasive strains have become more invasive, possibly by homologous recombination, clonal expansion of a previously rare clone or due to changes in the niche from reduction/re-ordering of other colonising species. Some strains are more likely to be invasive strains and an invasive strain is more likely to create a more favorable environment to ensure invasion of the host, this may be an evolutionary driver to exchange genetic material when co-colonisation occurs in a niche.

Table 1.1 Global burden of death in children less than 5 years of age due to invasive and non-invasive pneumococcal and Hib disease, 2008 (The World Health Organisation, 2012).

Disease	Global burden of death in children <5years of age, 2010
Pneumococcal pneumonia	167,600
H. influenzae type b pneumonia	183,700
Pneumococcal meningitis	45,300
H. influenzae type b meningitis	59,000

1.1.3. KEY PATHOGENS OF INVASIVE DISEASE

Much interest has been placed in *S. pneumoniae* genetics since Oswald Avery and his research team discovered that *S. pneumoniae* could induce transformation of DNA from a heat-killed organism in 1944 (Avery et al., 1944). Fifty-seven years later and the whole genome of *S. pneumoniae* TIGR4 strain was published with the ambition to discover novel vaccine targets and the mechanisms regarding the pathogenicity of *S. pneumoniae* (Tettelin et al., 2001). Individuals become colonised with *S. pneumoniae* and other nasopharyngeal flora during their first few months of life. The age of pneumococcal colonisation varies and may be attributed to environmental factors such as having siblings, attending daycare or geographical location (Aniansson et al., 1992, Principi et al., 1999). Colonisation with a pneumococcal isolate is a pre-requisite for pneumococcal infection; the capsule type of *S. pneumoniae* rather than genotype modulates the degree of infection (Brueggemann et al., 2003). The duration of colonisation in a child is dictated by the capsular type of the pneumococcus, and the rate of immune mediated clearance

increased as the age of the child increases (Lipsitch et al., 2012). *H. influenzae* is a Gram-negative rod-like human pathogen, and like *S. pneumoniae*, it occupies the nasopharyngeal niche. *H. influenzae* colonises infants during their first year of life in a similar manner to *S. pneumoniae* (Aniansson et al., 1992). Increased rates of *M. catarrhalis*, *S. pneumoniae* and NTHi acquisition during the first 12 months of infancy have been demonstrated to be indicative of whether an infection may develop in the form of OM (Faden et al., 1997), OM may have single or multi species causes. OM by *S. pneumoniae* alone was found to be caused by serotypes with greater invasive potential than OM by a mix of *S. pneumoniae* and NTHi, which was more likely to be caused by a pneumococcal serotype with less invasive potential (Dagan et al., 2013). These findings indicate that NTHi and *S. pneumoniae* co-carriage in children may add to the burden of otitis media.

1.1.4. MONITORING BACTERIAL AND PNEUMOCOCCAL CARRIAGE

For a bacterium to cause infection in a host, it must first be able to colonise that host (Ghaffar et al., 1999). There are studies, both completed and on-going, that monitor bacterial carriage in individuals (Tocheva et al., 2013, Hussain et al., 2005, Adegbola et al., 2014, van der Linden et al., 2015). Bacterial carriage may be monitored to detect changes following the implementation of preventative therapeutics such as a vaccine (Andrews et al., 2011), carriage studies are also useful for gaining baseline information such as carriage rates pre-therapeutic treatment. Carriage can be monitored for changes attributed to age, health status, geographical location, ethnicity and many other environmental factors. Carriage of a number of bacterial species can be monitored or carriage of a single bacterial species. Respiratory bacteria can be detected using relatively non-invasive means

such as a nasopharyngeal (Gladstone et al., 2012) or nose swab, meaning that larger numbers of patients can be recruited to strengthen the results gained from the study.

Pneumococcal carriage studies are highly informative and beneficial for a number of reasons. Firstly they can be used to monitor the carriage of *S. pneumoniae* serotypes pre- and post-pneumococcal conjugate vaccination (PCV) implementation. Through surveillance of invasive disease studies we have seen how PCVs are effective in reducing invasive VT pneumococcal disease (Flasche et al., 2011), and through carriage studies we can see the reduction of VT pneumococcal colonisation and VT pneumococcal transmission (Klugman, 2001). It is also possible to monitor the indirect effects of PCVs, such as changes in dynamics of the nasopharynx to detect microbial shifts in bacterial populations residing in the nasopharynx (Biesbroek et al., 2014). A valuable aspect of a pneumococcal carriage study is to determine whether serotype replacement has occurred and if it is exclusive to a certain demographic of the population being sampled, following introduction of a PCV.

1.2. PREVENTION IS BETTER THAN CURE

1.2.1. VACCINATION TO REDUCE DISEASE BURDEN

The 23-valent pneumococcal polysaccharide vaccine (PPV23; Pneumovax II™, Aventis Pasteur®) is a polysaccharide (PPS) vaccine. This type of vaccine induces an immune response to the polysaccharide (PS) capsule of an infectious organism to induce short-term memory B-cells and antibody production. PS antigens in a PPS produce a T-cell independent response (Weintraub, 2003) and are processed by B-cells who do not require antigen presenting cells (APCs) as B-cells

recognize the PS antigen in its natural form (Clem, 2011). PPS vaccines elicit slow immune responses with no immune memory; this is not effective in young children and infants (both of whom are IPD risk groups). Immunological memory calculated by serum antibody levels was found to last less than 6 months, post PPS vaccination, in children 18 months old or younger (Kayhty et al., 1984). T-independent vaccine responses are particularly poor in young children due to an infants undeveloped immune system, including an immature spleen marginal zone (Timens et al., 1989), and PPS vaccines won't prevent carriage of the bacterial species (Douglas et al., 1986) after the short-lived immune response has finished.

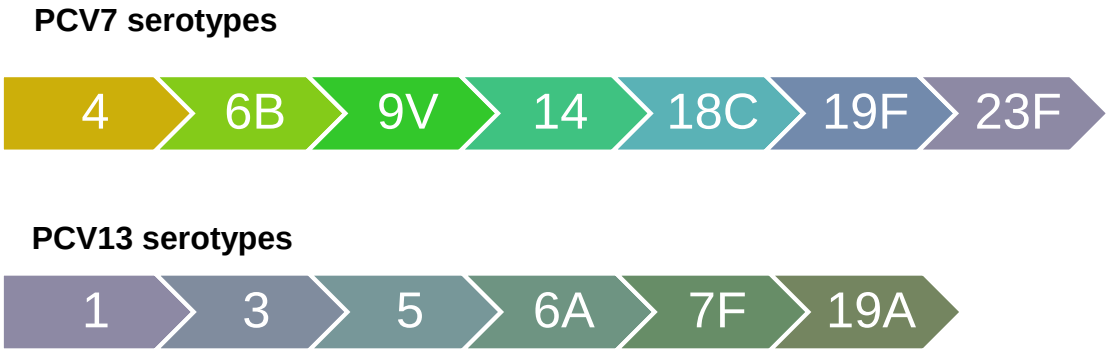
The seven-valent pneumococcal conjugate vaccine, PCV7; Prevenar™ Pfizer®) contains bacterial polysaccharides from the outer capsule of an organism which are conjugated to diphtheria toxoid protein. This allows the immunogenic properties of the carrier protein to be conferred to the polysaccharides, thus creating an effective immunogen. The polysaccharide is converted into a T-dependent antigen through the presence of the carrier protein. Long-term memory B cells mature so that the immune system has both a short-term and long-term response invoked when those polysaccharides are encountered again. This reduces colonisation of the serotypes included within the vaccine, helping to prevent infection. PCVs are given to children rather than a PPV because the conjugated protein in a PCV, mounts a stronger and long-lasting immune response (National Health Service, 2010). Recommendations have been made in support of using PCV only in adults as vaccination with PCV followed by PPV increases the risk of PCV hyper-responsiveness (de Roux et al., 2008).

FIGURE 1.1 CONJUGATE VACCINES CURRENTLY INCLUDED IN THE UK PAEDIATRIC IMMUNISATION SCHEDULE

1.2.2. PNEUMOCOCCAL CONJUGATE VACCINES

PCV formulation was developed based upon the prevalence of pneumococcal serotypes in the 1990s. PCV7 includes seven invasive disease-causing serotypes (Table 1.3) that were responsible for 80% of invasive infections in US children in 1998 (Advisory Committee on Immunization, 2000, Paradiso, 2011). Globally those seven serotypes were also found to cause the majority of invasive disease (Johnson et al., 2010). PCV7 was added to the UK paediatric national immunisation program (NIP) in September 2006 (National Health Service: Local, 2012) and PCV13 (Prevnar 13™, Pfizer) was introduced to the UK in April 2010 (National Health Service: Local, 2012). PCV13 contains the serotypes of PCV7 as well as six additional serotypes (Table 1.2) seen to cause considerable disease within both Europe and North America (Centres for Disease Control and Prevention, 2010). These serotypes were included because they either a) caused disease due to a serotype shift seen post-PCV7 or b) in addition to causing disease in Europe and North America, they were also causing considerable disease in developing countries, such as certain Asian regions or Sub-Saharan Africa (Centres for Disease Control and Prevention, 2010).

FIGURE 1.2 PCV FORMULATIONS PREVIOUSLY AND CURRENTLY USED IN THE UK



In the UK, infants are routinely vaccinated in an attempt to prevent invasive and non-invasive pneumococcal disease. A pneumococcal conjugate vaccine is included in the paediatric NIP (National Health Service: Local, 2012) (Table 1.3.), and is given as a series of vaccinations to children aged between 2-13 months.

Table 1.3 UK Paediatric immunisation schedule, 2012 (National Health Service, 2012)

Age for immunisation:	Vaccination to be received:
2 months	o 5 in 1 (DTap/IPV/Hib) – Diphtheria, tetanus, polio, pertussis, *H. influenzae B.* o PCV13 pneumococcal vaccine
3 months	o 5 in 1 - second dose o Meningitis C
4 months	o 5 in 1 - third dose o PCV13 pneumococcal vaccine (second dose) o Meningitis C - second dose
12-13 months	o Hib/Men C booster o MMR (measles, mumps & rubella) o PCV13 pneumococcal vaccine (third dose)
3 years & 4 months	o MMR second dose o 4 in 1 pre-school booster (DTap/IPV)
12-13 years	o HPV cervical cancer vaccine (girls)
13-18 years	o 3 in 1 teenage booster (Td/IPV)

GSK has produced a 10-valent pneumococcal vaccine – PHiD-CV10 (Synflorix™) that differs from PCV7 and PCV13 by the binding of 10 pneumococcal serotypes each to one of three protein components, namely NTHi protein D, tetanus toxoid and diphtheria toxoid. PCV10 has not been included in the UK paediatric NIP but is licensed for use in both the UK and in a number of countries. A study has shown that PCV10 is comparable to PCV7 at preventing invasive pneumococcal disease (Palmu et al., 2013) however, PCV13 was introduced to the schedule due to the increased incidence in replacement IPD caused by additional serotypes found in the PCV13 formulation but not in PCV7 or PCV10 (JOINT COMMITTEE ON VACCINATION AND IMMUNISATION, 2009).

Many pneumococcal vaccination studies are primarily focused on young children and infants. However, recently these studies have been expanded to include adults and older adults as the immune responses differ through the age of an individual. In terms of pneumococcal vaccinations for adults in the UK, PPS vaccines are used if an individual falls into a 'risk-group'. Risk groups include those with chronic conditions and those over-65 years of age (Public Health England, 2014b) In 2012, it was demonstrated that the PCV7 vaccine is more effective in inducing memory B-cell production than PPV23 in both the child and adult groups (Clutterbuck et al., 2012). Trials have been carried out to assess the efficacy of PCV13 in older adults (Pfizer, 2013) reporting an increased immune response with PCV13 administration when compared to PPV23 administration in both unvaccinated adults and adults previously vaccinated with a plain polysaccharide vaccine (Paradiso, 2012).

PCV effectiveness is subject to strains undergoing capsular changes including; serotype replacement/shifting (Spratt and Greenwood, 2000) – where prevalence of a non-vaccine serotype increases as prevalence of a vaccine-serotypes decreases and the non-vaccine type bacteria overcome vaccine

challenges in a community (Jefferies et al., 2004), and capsular switching - where an individual bacteria can undergo changes in the capsular genes, causing the bacteria to change serotype (Wyres et al., 2012). Through alteration of capsular expression and the increase in prevalence of serotypes not included in vaccine formulations, serotypes in carriage may be replaced with more virulent serotypes (Porat et al., 2004). However it has been estimated that capsular switching resulting from vaccine pressures will only contribute to an increase of a maximum of three extra cases of IPD per 100,000 vaccinated children per a year cumulated over a ten-year period. The additional maximum of three cases per a year was deduced using a mathematical model of pneumococcal transmission based on IPD data presented in previous European publications (Temime et al., 2008). However, until there is evidence from more studies of capsular switching post PCV, IPD from non-vaccine type pneumococci may be a more pressing issue (Munoz-Almagro et al., 2008).

Antibiotic resistance in pneumococcal isolates has been shown to be globally present in both carriage (Kumar et al., 2014, Pimentel de Araujo et al., 2014, Lee et al., 2014) and disease (Goldsmith et al., 1997, Navarro Torne et al., 2014, Cho et al., 2014). Reasons for pneumococcal antibiotic resistance include vaccine pressures as well as over-prescribing and over-use of antibiotics acting as a selective pressure for current strains to undergo clonal expansion (Keenan et al., 2014, Song et al., 2012).

The focus of future pneumococcal vaccine research is beginning to be placed on common protein vaccines. A vaccine would contain single or multiple proteins that would be essential to the pathogenesis of *S. pneumoniae,* a protein vaccine would not need to rely on the inclusion of tens of polysaccharides and would remain unaffected by changes in capsule (Program for Appropriate Technology in Health, 2012b). There are difficulties in obtaining a protein suitable for vaccine inclusion, as proteins must be deliverable with low adverse effects whilst providing adequate protection from *S. pneumoniae* strains. Some proteins that are currently being trialed

include pneumococcal virulent factor pneumolysin (*Ply*) derivatives, the polysaccharide capsular protein A (*PcpA*) and histadine protein D (*HpD*) (Vadesilho et al., 2012, Kamtchoua et al., 2013, Seiberling et al., 2012). These candidates are undergoing further testing with results indicating that the vaccines can provide protection from invasive infection in mice (Verhoeven et al., 2014). Another potential vaccine candidate is an inactivated whole cell vaccine, such as that currently being trialed through the partnership between PATH, Boston Children's Hospital and Brazil's Instituto Butantan (Program for Appropriate Technology in Health, 2012a). Results from vaccinated mice indicate that the vaccine is effective at preventing pneumococcal carriage and prevents against pneumococcal sepsis (Lu et al., 2010). A key requirement of next generation pneumococcal vaccines is being able to mount an effective immune response using noncapsular proteins whilst being cost effective to produce,, so far this has been shown in murine models and the results from human trials will determine if these vaccines have the potential to replace capsular based vaccines (Moffitt and Malley, 2011).

1.2.3. *HAEMOPHILUS INFLUENZAE* B VACCINATION

Before the inclusion of the Hib conjugate vaccine in the UK paediatric NIP in 1992 (Meningitis UK, 2012), around 95% of invasive *H. influenzae* disease was attributed to serotype b alone (Turk, 1984). After widespread use of Hib, a dramatic 98% decrease in invasive disease was observed by 1998 (Heath and McVernon, 2002). However, Hib disease has still been reported in some vaccinated populations due to vaccine failure in the UK and elsewhere (Heath and McVernon, 2002, Galil et al., 1999). Whereas invasive Hib presented in young children, most invasive

Haemophilus disease is now caused by NTHi, particularly in the adult and elderly populations (Adam et al., 2010, Shuel et al., 2011).

The increasing antibiotic resistance within *H. influenzae* implies that genetic recombination is taking place within the species, with antibiotic resistance genes being incorporated into the genomes of NTHi isolates (Shuel et al., 2011). Increased numbers of antibiotic resistant strains may not be the only result of increased genetic diversification; diversification may also impact their ability to cause disease. With predominant carriage of NTHi strains, it is expected that there will be an increase in disease incidence of NTHi as bacterial carriage is a pre-requisite for disease. With this, the development of a vaccine against NTHi becomes a priority. An investigational vaccine for NTHi is currently undergoing clinical trials in a cohort of people with COPD, the type of vaccine is currently unknown but recruitment of patients has begun (GlaxoSmithKline, 2014).

1.2.4 IMMUNISATION INEQUALITIES

According to the research and work carried out by GAVI, the Vaccine Alliance and the International Vaccine Access Centre, Hib was first introduced into a high-income country NIP in 1986. Eight years later in 1994, Hib was first introduced into a middle-income country NIP.. Hib was then introduced into a low-income country NIP three years later in 1997, a total of eleven years after it was first introduced to a high-income country NIP. As of 2014, globally 30% of infants still live in countries where Hib vaccine has yet to be introduced to their NIP (International Vaccine Access Centre, 2014).

PCVs were introduced into a high-income country NIP in 2000. It took another eight years before a PCV was introduced into a middle-income country NIP in 2008. One year later it was introduced into the immunisation of a low-income country NIP in 2009. As of 2014, globally 59% of infants still live in countries where a PCV vaccine has yet to be introduced to their NIP (International Vaccine Access Centre, 2014).

1.3. THE NASOPHARYNX AS A MICROBIAL NICHE

1.3.1. INTERACTIONS OF NASOPHARYNGEAL MICROBIOTA

The microbiota of the human nasopharynx contains both commensal and potentially pathogenic species with external environmental factors and the presence of antibiotic resistant species contributing to disease-states (Leiberman et al., 1999).

Bacteria found to reside in the nasopharynx include *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, non-pneumococcal alpha-haemolytic streptococci (αHS) including *Streptococcus viridians*, *Staphylococcus aureus* and *Neisseria meningitidis*. *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. aureus* and *N. meningitidis* have been included in this study as they are all respiratory bacteria capable of causing significant infections. Bacteria that phenotypically displayed qualities of other species of alpha haemolytic streptococci (αHS) were stored awaiting further characterisation as many relations of S. pneumoanie are known to be closely related, requiring 16S rRNA analysis to differentiate (Kilian et al., 2008). *M. catarrhalis* is a non-motile gram-negative human commensal and opportunistic pathogen responsible for a range of infections, including causing an estimated 10% of COPD exacerbations (Murphy et al., 2005). It has been shown that even the colonisation of *M. catarrhalis* in a COPD patient can contribute to the progression of

airway disease (Parameswaran et al., 2009). Streptococci are a genus of gram-positive species of bacteria that can be commensal, pathogenic or opportunistically pathogenic. They are sub-divided into many groups based upon their physical properties and their similarities when using microbiological typing methods. Firstly they are divided according to their type of haemolysis; α-haemolytic, β-haemolytic and γ-haemolytic streptococci. β-haemolytic streptococci are further typed using the Lancefield (Lancefield, 1933) grouping based on capsular polysaccharides into groups A to H, group A streptococci include *Streptococcus pyogenes*, responsible for 'strep throat'. *S. pneumoniae*, *Streptococcus viridians*, S*treptococcus mutans* and *Streptococcus sanguis* are all α-HS. *S. pneumoniae* are Gram-positive diplococci often found to occupy the nasopharyngeal niche. Pneumococci are typed according to the serological response to their external polysaccharide capsule. Strains of *S. pneumoniae* that do not react with type-specific antisera are deemed nontypeable (NT) *S. pneumoniae*. Currently 94 pneumococcal serotypes have been characterized (Rodgers and Klugman, 2011, Bratcher et al., 2010, Calix and Nahm, 2010, Henrichsen, 1995, Park et al., 2007, Calix et al., 2012). *S. aureus* is a gram-positive cocci-shaped bacterium. It is also an opportunistic pathogen that causes a wide range of infections from non-serious skin infections to invasive bacteremia and sepsis. It is plays an important role in wound infections, particularly in healthcare-associated- infections. *S. aureus* is broadly split into two groups based on its susceptibility to meticillin, namely meticillin-sensitive *S. aureus* (MSSA) and meticillin-resistant *S. aureus* (MRSA). It is important to assess the carriage of *S. aureus* and to monitor if carriage of MRSA is increasing, as it has become a significant cause of healthcare-associated infections in recent years, with patient transfers helping spread the resistant clones from area-to-area (McAdam et al., 2012). *N. meningitidis* is a gram-negative diplococcus causing meningitis and septicemia. It is subtyped into 12 groups based on its capsular sugars. Carriage of *N. meningitidis* in children is higher at around 1 year of age and after 15 years of age

(Bogaert et al., 2005). *H. influenzae* are Gram-negative coccobaccilli, Serological typing based on the polysaccharide composition of the capsule, is used to classify *H. influenzae* and there are a total of six serotypes; (a – f), as well as a large, distinct population (Meats et al., 2003b) that are unencapsulated and are termed nontypeable *H. influenzae* (NTHi). In addition to causing disease, both organisms are commonly found to colonise the upper respiratory tract in children. The above bacteria have demonstrated that they have the ability to cause acute infections in humans after colonising the nasopharynx and this makes them clinically relevant in this study.

1.3.2. NASOPHARYNGEAL MICROBIAL CHARACTERIZATION

One of the techniques being used by researchers that are interested in determining all bacterial carriage from a single site is that of 16S rDNA gene community profiling. Using a sequence common to all eubacteria, a fragment of DNA present in all strains can be detected and amplified from DNA extracted from a mixed source, in a non-selective manner. The bacterial sequences that have been amplified and sequenced are divided according to their phylogenetic groups of *Actineobacteria, Proteobacteria, Fermicutes* and *Bacteriodetes* and can be assigned to the level of the bacterial genus. This technique has been used widely in environmental microbiology (Marchesi et al., 1998) and also now as a means of comparing bacterial niches in the human body (Lemon et al., 2010). 16S-sequencing technology on the Roche platform has been used to sequence nasopharyngeal swabs of children with or without otitis media. This study aimed to determine the relationships of bacteria and investigate whether there is a difference in the microbiota between two groups of children and demonstrated that invasive infection

is associated with increased *S. pneumoniae* and *H. influenzae* being present with a lack of protective flora present (Laufer et al., 2011). Studies such as this add to the broader epidemiological literature by demonstrating what genera of bacteria are commonly carried in the nasopharynx, as well as elucidating the bacterial dynamics of strains within a niche to help describe the human microbiome. One study set out to characterize the nasopharyngeal niche to deduce which, if any, external factors (such as viral carriage and day-care level) have an effect on the microbial make-up of the nasopharynx in children less than seven years of age, results showed that seasonal changes were occurring but that these were unrelated to viral or antibiotic causes. The resulting observation was that seasonal variations corresponded to 'healthy' pro-biotic species being more abundant in summer rather than autumn (Bogaert et al., 2011). These individual studies contribute to the Human Microbiome Project, a series of ongoing studies that combine to elucidate the composition of our microbial components and the effects they have on our physiological make-up (Turnbaugh et al., 2007).

1.3.3. A NICHE OF CARRIAGE AND INFECTION

Bacterial interactions within the nasopharynx can be considered as being dynamic as they comprise of both synergistic and competitive associations. A synergistic association is where two or more factors that work together so that the total effect is greater than the sum of the involved factors. A competitive association is where two or more factors compete directly against each other in order for one factor to be more successful than the other. These associations can change depending whether or not the niche is in a healthy or a disease-state (Xu et al., 2012a), research has shown that a lower diversity of nasopharyngeal flora is

positively associated with higher carriage rates of nasopharyngeal pathogens including *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (Pettigrew et al., 2012).

External factors such as sibling interaction and interaction with other children can play a part in polymicrobial carriage and these factors are positively associated with more frequent nasopharyngeal carriage of potential pathogens (Garcia-Rodriguez and Fresnadillo Martinez, 2002). Adult associations with more frequent carriage of potential pathogenic species include, but are not limited to, the presence of children either at home or at work, pre-existing allergic conditions and COPD (Garcia-Rodriguez and Fresnadillo Martinez, 2002). This implies that children are reservoirs for bacterial pathogens, and with increased contact between children and other children or children and adults, there is a greater chance for bacterial transmission between the individuals

S. pneumoniae and *H. influenzae* cause similar infectious diseases and they occupy the same niche, the nasopharynx. Frequently found to co-colonise together, competition may exist between these two organisms for nutritional resources and hence for dominance of the niche. It has been shown that *H. influenzae* when colonising with *S. pneumoniae* may out-compete it for survival through signaling of nucleotide-binding oligomerisation domain-1 (Nod1) to facilitate clearance of *S. pneumoniae* (Lysenko et al., 2007), but virulent *S. pneumoniae* serotypes show resistance to host cell-mediated clearance as a mechanism to overcome these attacks (Lysenko et al., 2010). Both organisms cause immune responses in colonised individuals and co-colonisation by these two pathogens can result in exaggerated immune responses with prolonged hospitalization particularly for young asthmatics experiencing their first count of wheeze (Jartti et al., 2011).

A polymicrobial infection can result in both acute and chronic conditions, caused by a combination of several microorganisms. A chronic polymicrobial

infection can be described as a long-term situation where eradication of the infectious agents is not realistically achievable and treatment will be to manage the effects of the infection (Rogers et al., 2010). An acute polymicrobial infection is a short-term infection that is cleared using an appropriate treatment. The colonisation of one species in an area can help to create an ecological niche by encouraging other species to also colonise. Bacteria do this by either creating a more favorable environment or interfering with host-immune responses, co-colonising pathogens can include bacteria, fungi and viruses (Brogden et al., 2005). A polymicrobial infection combining both bacteria and fungi can result in a greater immune response within a host than infection by either bacteria or fungi (Peleg et al., 2010). Another method of infection occurs when a bacterial pathogen attaches to a surface and forms a biofilm, this is where the usually planktonic bacterial cells aggregate together and produce a extracellular matrix made up of polymers to protect itself from host immune responses and increase persistence and survival within a host (Costerton et al., 1987, Hall-Stoodley et al., 2004). It is possible for one or several non-pathogenic species to colonise together to create a pathogenic biofilm (Peters et al., 2012). Within a biofilm is just one way that bacteria can co-colonise together to enhance their survival and evasion of host immune responses, while co-colonising it is possible for strains of bacteria to share genetic information with each other.

Reduced clonality within a species means that strains are less similar to each other than a species with high clonality, *H. influenzae* is a genetically diverse organism and modern molecular techniques demonstrate the reduced clonality of NTHi compared to capsulated isolates (Sacchi et al., 2005) NTHi vaccine advances may be short-lived if the strains continue to diversify. The distributed genome hypothesis described for *H. influenzae* and *S. pneumoniae* (Hogg et al., 2007, Hiller et al., 2007, Boissy et al., 2011) indicates that the entire genome of a bacterial species is distributed throughout its various strains, allowing for acquisition of genetic

material between strains when they colonise together. The idea of non-core sequences being available for genetic recombination, providing an available sup*ply* of sequences to aid in genetic diversification can explain how these two pathogens may display more virulence when co-carried. Antibiotic resistance and virulence traits can be transferred through homologous recombination from other strains as seen with *S. pneumoniae* (Mostowy et al., 2014), particularly when they co-exist in a microbiome.

1.3.4. INDIRECT EFFECTS OF PCV IMPLEMENTATION

Pneumococcal conjugate vaccines (PCVs) affect the carriage of *S. pneumoniae* and the carriage of vaccine type (VT) serotypes. A large study of healthy children and children with re-current otitis media, all less than 36 months of age in Western Australia has shown that with a decrease in *S. pneumoniae* and *S. pneumoniae* PCV7 VT serotypes there is a corresponding increase in *H. influenzae,* particularly NTHi (Wiertsema et al., 2011). This result adds weight to the hypothesis that a dynamic relationship exists between these two organisms (Lysenko et al., 2005, Shakhnovich et al., 2002, Ratner et al., 2005). The eradication of PCV7 VT in the nasopharynx has also been associated with higher rates of *H. influenzae* and *S. aureus* carriage in young children and infants, highlighting that virulent serotypes of *S. pneumoniae* also have a competitive relationship with *S. aureus* as well as *H. influenzae* (Spijkerman et al., 2012). It is important to note that some conclusions made by researchers in this field are based upon the methodology of standard microbiology culture. This can bias results by selectively excluding difficult-to-culture organisms and those present at lower concentrations, however bacterial carriage monitoring through microbiological culture allows researchers to then type the

organisms by both PCR and MLST. Incidences of both *S. pneumoniae* and *H. influenzae* being carried together in an individual should be recorded to inform future vaccine policy. This is important as the effect of vaccines targeting either of these two opportunistic pathogens may produce indirect effects as these two species share similar properties and the ultimate balance between the two organisms is unknown.

1.4. USING MOLECULAR METHODS TO INFORM VACCINE POLICY

1.4.1. MOLECULAR TYPING

In addition to being useful for investigating disease outbreaks and determining transmission dynamics (Harrison et al., 2011), molecular epidemiology is also useful in a varied bacteria-rich environment, where the specific organism or strain of organism responsible for a given case or outbreak of disease may not be clear. Epidemiological studies may be for singular or multi species pathogens or for identification of a strain, serotype or clone of interest. Total community analysis such as characterizing a microbiome, independent of microbiological culture, is now also possible using 16S rDNA microbial community sequencing (Costello et al., 2009)

The molecular typing approaches of polymerase chain reaction (PCR) and multi locus sequence typing (MLST) are now widely used for disease surveillance and research of both *S. pneumoniae* and *H. influenzae*, as the epidemiology of strain prevalence and distribution is built when compared to carriage studies of other populations and locations. The use of MLST allowed all isolates, including non-typeable (by PCR) isolates of a species to be assigned a sequence type (ST). Any changes in prevalence of serotypes and sequence types can be observed over time (Tocheva et al., 2011). MLST of *S. pneumoniae* was first described by Enright and

Spratt (Enright and Spratt, 1998). MLST is used to assess the clonality between strains and isolates. A ST is assigned to encapsulated and unencapsulated strains of *S. pneumoniae* and *H. influenzae* allowing them to be typed based on genetic relatedness. When using phenotypic serotyping methods, some isolates, due to a lack of expression of capsular antigens, cannot be assigned a serotype and are then deemed as being an 'untypeable' strain. MLST can be used to identify culture negative organisms, such as *H. influenzae* and *S. pneumoniae* from middle ear fluids, by analyzing the samples direct from a source when otitis media is suspected but no bacteria is cultured (Xu et al., 2011). Antibiotic resistance, particularly penicillin resistance in *S. pneumoniae* can be indicative of the expansion of a particular ST or clonal complex, rather than serotype (Henriqus Normark et al., 2003). Singular pathogen PCR was used in this study to estimate the prevalence of PCV13 VT and PCV13 NVT serotypes of carried *S. pneumoniae* in a sample population group. MLST was utilized in this project for assigning a ST to the isolates used in this study.

1.4.2. WHOLE GENOME SEQUENCING

Whole genome sequencing (WGS) is the assembly of the entire genome sequence of an organism. This can be achieved by reference to an existing genome sequence for the organism (targeted re-sequencing) or by assembling sequence fragments *de novo*.

High throughput WGS methods are becoming more widely used and affordable, and are being applied to bacterial genomics. These techniques are able to characterize bacterial strains in greater detail than standard molecular microbiology and genetic differences can be elucidated for large numbers of strains.

De novo sequencing was used in this study for genomic analyses of carriage strains of *S. pneumoniae* and *H. influenzae*.

Much has changed from the first time the complete bacterium of *H. influenzae* Rd was sequenced in 1995 by Fleischmann *et al* (Fleischmann et al., 1995). Interesting results have been produced on the comparison of bacterial strains of *S. pneumoniae* (Hiller et al., 2007) and *H. influenzae* (Hogg et al., 2007) and using these new gene clustering algorithms to aid comparisons of genome sequencing between different bacterial organisms (Boissy et al., 2011). We have entered an era of bench-top bacterial sequencing with increased numbers of companies producing personal sequencing machines that are proving very effective in the whole genome sequencing of bacterial strains; however large scale sequencers are required for cost-effectiveness and time demands when dealing with increasing numbers of strains requiring sequencing.

There are many uses of a bacterial genome sequence, from determining the evolutionary history of a strain, an outbreak investigation, protein expression and determining virulence factors. The field of reverse vaccinology, pioneered by Rino Rappouli and colleagues, is based on the use of whole genome sequencing to identify vaccine candidate molecules (Rappuoli, 2001) as an alternative approach to using vaccine components found to be immunogenic from serological evidence (Wizemann et al., 2001). With increasing numbers of laboratories now using bench-top sequencers, the next stage is to develop WGS as a routine diagnostic and surveillance tool (Long et al., 2013). Typing and characterisation of samples can be achieved through *in silico* methods derived from the traditional typing methods. PCR and MLST can be carried out *in silico* using the bioinformatics program Short Read Sequence Typing 2 (SRST2), (Inouye et al., 2012). SRST2 combined with access to a particular database, such as an MLST database can be used for analyzing whole genome sequences of the microorganism to give fast and accurate typing results. It

is also possible to manually curate a database to type for certain genes of interest such as antibiotic resistance and virulence genes.

1.5. POLICY-MAKING

In the UK, the Joint Committee for Vaccination and Immunisation (JCVI) (JCVI, 2014) advise UK health departments on matters relating to immunisation, meeting three times a year to discuss the costs and benefits of different vaccines and advise which should be added to the UK NIP. JCVI reviews information gained from carriage studies and disease surveillance in the UK and worldwide that focus on the effects of a particular vaccine and along with the cost benefit, advise on whether it should be used in the UK schedule. The JCVI has recently reviewed their decision to include PCV13 in the UK immunisation schedule opposed to PCV10. After reviewing carriage study and invasive disease data, the JCVI recommended to retain PCV13 as part of the UK NIP as studies have shown that PCV13 was more effective at reducing invasive disease than PCV10 due to the extra serotypes included in its formulation. However, serotype replacement data remains inconclusive (JCVI)

1.6. SUMMARY

Although there has been much research in the field of *S. pneumoniae* and *H. influenzae* dynamics there is still a gap in our understanding of these populations at a genomic level in the nasopharyngeal niche. This study describes the molecular epidemiology of these pathogens in the post-PCV era. In this study, the bacterial dynamics of the nasopharyngeal niche will be explored during PCV13

implementation, with a focus on the interactions of the various serotypes and genotypes of these bacterial species. Ultimately this study will contribute towards informing vaccine policy by reporting current pneumococcal UK carriage data results and determining relationships of bacteria carried in the nasopharynx during PCV7/PCV13.

1.7. STUDY RATIONALE

PCV7 was added to the UK as part of the NIP in 2006. The effect of PCV on pneumococcal carriage has been investigated (Tocheva et al., 2011). A study was developed to monitor pneumococcal carriage and the carriage of PCV7 VT serotypes in the nasopharynx of young children aged 4 years and under (REC: 06/Q1704/105). The study began in the winter season of 2006/07 initially with the culture of *S. pneumoniae* only, but from 2008/09 the study was expanded to involve monitoring of other clinically relevant bacteria that reside in the nasopharynx. The data shown in this thesis is based upon the information gained from individual and multi-bacterial analysis of carried isolates from this study.

This study aims to describe the changing microbial epidemiology in the nasopharynx of young children following pneumococcal vaccine introduction. It has been shown that some PCV7 VTs vaccine engaged in mechanisms to overcome the effect of the vaccine including capsule-switching and genetic drift. Vaccine escape occurs to differentiate a strain from their previous serotype included within the pneumococcal vaccine (Wyres et al., 2013, Croucher et al., 2013). PCV13 was required due to PCV7 NVTs increasing in prevalence as additional serotypes were found to be responsible for causing IPD (Kyaw et al., 2006).

In the UK, as elsewhere, the incidence of IPD has decreased after the implementation of PCV's (Miller et al., 2011) and as additional higher valent PCVs are introduced it is expected that IPD incidence will continue to decrease (Choi et al., 2012). The effect of pneumococcal vaccination on other bacterial species known to occupy the same niche as *S. pneumoniae* has not been fully investigated. Research can now be carried out to determine whether changes in the human microbiome can be attributed directly or indirectly to external pressures or vaccine introductions (Turnbaugh et al., 2007, Peterson et al., 2009). A study has shown that where VTs of *S. pneumoniae* have been eradicated, there has been an increase in non-typeable *H. influenzae* being isolated in cases of otitis media (Wiertsema et al., 2011). There could be several explanations of this increase, all needing to be investigated. For example, the removal of an infectious pathogen – i.e. PCV13 vaccine serotypes, could encourage opportunistic bacterial serotypes to compete for increased colonisation rates. Before the widespread use of vaccines, bacterial colonisation and infection was in part, controlled by the dominant pathogen present, this can be seen in recent research where the PCV7 vaccine was associated with an increase in colonisation of non-pneumococcal species of bacteria (Spijkerman et al., 2012). Research has also highlighted that colonisation with invasive serotypes of *S. pneumoniae* is associated with a decrease in colonisation of *H. influenzae* (Xu et al., 2012b).

1.8 THESIS HYPOTHESIS

It is proposed that, following implementation of PCV13, there will be a decrease in the prevalence of vaccine-type pneumococcal serotypes carried in the nasopharynx of young children, accompanied by an increase in carriage of non-vaccine serotypes. The highly transformable *H. influenzae* will be investigated to determine if corresponding changes are detectable at the level of carriage and genetic content.

1.8.1 PRIMARY AIM

- To combine culture-based and molecular typing of *S. pneumoniae* and *H. influenzae*, with whole genome sequencing to investigate genomic content and relatedness of isolates in carriage after the introduction of pneumococcal conjugate vaccines. Evidence of single nucleotide polymorphisms (SNP) and recombination will also be investigated for both species of bacteria.

1.8.2 SECONDARY AIM

- To identify possible virulence factors and their abundance within a species to determine their potential as markers for novel therapies.
- To identify *in silico* antibiotic resistance within carriage isolates of *S. pneumoniae* and *H. influenzae*.

2. MATERIALS AND METHODS

2.1 STUDY SET-UP

This study is an on-going population based cross-sectional study that primarily focused on the nasopharyngeal carriage of *S. pneumoniae* in children four years of age and under. Parents of eligible children were approached when they were attending the paediatric outpatients' clinics at the University Hospital Southampton. Between 2006/07 and 2007/08), the study was designed for detecting pneumococcal carriage following PCV7 introduction to the UK NIP. As a new potential vaccine was announced (PCV13) it became important to identify other respiratory bacteria carried in the nasopharynx, so clinically relevant bacteria were then chosen for inclusion in years 2008/09 and 2009/10 onwards of the study, listed in Table 2.1. Parents were asked to fill out short questionnaires regarding the child's vaccination status and recent health/antibiotic use from years 4 of the study onwards.

2.1.1 STUDY STATEMENT

The carriage study as described in Chapter 1.6 was carried out as described by Tocheva *et al* (Tocheva et al., 2011). The laboratory analysis of pneumococci isolated from carriage study swabs described in section 2.1.7 was carried out in part by Anna Tocheva or Rebecca Gladstone (both were PhD students at the time the work was carried out) and I assumed this work from 2011/12 onwards. I carried out all other work, where technical assistance was used; it is stated clearly in the relevant methods section. Use of external companies/services throughout this study is clearly stated in the relevant chapter sections.

2.1.2 STUDY SETTING AND PARTICIPANTS

Nasopharyngeal swabs were collected from children aged four years and under attending the paediatric outpatients department between October to April during each of the study years (Table 2.1). Only one sibling per family was swabbed to avoid bias from family members that may have similar colonisation patterns. Participants may have been attending any clinic but no single clinic was targeted preferentially. Children attending a clinic regarding cancer or immunosuppressive therapy were excluded from this study. Children were recruited after gaining informed consent from a parent or guardian. Inclusion was regardless of health and vaccination status, gender or ethnicity (Tocheva et al., 2011).

2.1.3 NASOPHARYNGEAL SWABBING

Samples were obtained by trained personnel (paediatric research nurse or trained medical student). A dry cotton-wool swab with flexible wire shaft (TRANSWAB, Medical Wire and Equipment, Corsham, UK) was inserted through one nostril in order to sample the nasopharynx. Swabs were placed immediately into Amies® charcoal transport medium. Swabs were sent immediately to the Health Protection Agency (HPA, now part of Public Health England) South East Region Laboratory on the same site and processed within six hours according to standard protocol. Swabs were plated onto Colombian blood agar (CBA), chocolate blood agar (CHOC), bacitracin agar (Bact), Colombian Naladixic Acid agar (CNA) and

vancomycin, collistin, amphotericin and trimethoprim (VCAT) plates and stored overnight at 37 degrees Celsius with 5% carbon dioxide.

2.1.4 ETHICAL APPROVAL

Ethical approval was granted (REC 06/Q1704/105) by the Southampton and South West Hampshire Research Ethics Committee B.

2.1.5 SAMPLE SIZE

In order to detect a 50% reduction from the lowest estimated pneumococcal carriage rate of 10% following the introduction of PCV7 to a 5% significance level with 80% power, a sample size of 100 pneumococci per study period was required based on the lowest expected carriage rate of 10%.

2.1.6 MICROBIOLOGY

Samples were plated onto selective medium and primary plates and examined after 18hr incubation, at 37°C with 5% CO_2, for the presence of pneumococci, and other selected bacterial species, see Table 2.1.If a primary plate showed colonies with a pneumococcal morphology, bacteria were subjected to sensitivity testing with an optochin disc before being classed as positive. Then ten individual colonies of pneumococci were sub-cultured and subsequently stored at -

80°C on cryogenic beads, (Microbank – Prolab) swabbing years 1 to 3 and in skim milk, tryptone, glucose and glycerin (STGG) during swabbing years 4 to 7 for future analysis to determine the frequency of carriage of multiple serotypes and sequence types. If present, colonies indicative of other species of interest listed in table 2.1 were cultured and then identified by a state registered biomedical scientist using routine laboratory methods. One of the positively identified colonies was then sub-cultured for purity and stored at -80°C on cryogenic beads (Microbank – Prolab). The swab head was aseptically cut into a vial of sterile BHI and 50% glycerol and stored for the future analysis of bacterial DNA only. As a result of this carriage study, there were an excess of 700 pneumococci detected and stored, over 300 *H. influenzae* isolates, around 400 *M. catarrhalis* isolates and several hundred original swab heads, as well as smaller numbers of *S. aureus*. This study produced a large and distinct set of UK bacterial isolates recovered from the nasopharynx that would benefit many other researchers in the field should it be curated into the form of a biobank for research purposes.

Table 2.1 Study years and bacteria included in study

Swabbing season	Year	Bacteria culture for in study
Year 1	2006/07	*S. pneumoniae*
Year 2	2007/08	*S. pneumoniae*
Year 3	2008/09	*S. pneumoniae, H. influenzae*, *N. meningitidis*, meticillin sensitive (MSSA) *S. aureus*, meticillin resistant (MRSA) *S. aureus*.
Year 4	2009/10	*S. pneumoniae, H. influenzae, N. meningitidis*, MSSA, MRSA, *M. catarrhalis.*
Year 5	2010/11	As year 4
Year 6	2011/12	As year 4 and 5
Year 7	2012/13	As years 4, 5 and 6

2.1.7 PNEUMOCOCCAL ISOLATES

Isolates of pneumococci were sub-cultured on Colombia blood agar (Oxoid, UK) before being used for DNA extraction as described in chapter 2.2. PCR-based capsular typing (2006/07 – 2010/11) was carried out by other members of the group using previously published protocols (Pai et al., 2006, Centres for Disease Control and Prevention, 2012, Tocheva et al., 2011). The data from this was made available for use in the current study in order to calculate the prevalence of *S. pneumoniae* serotypes included in PCV13. I typed pneumococcal isolates from study years 2011/12 and 2012/13. This method does not allow identification of all possible serotypes; therefore isolates that could not be typed by PCR were serotyped at the

Statens Serum Institute using factor-specific anti-sera (Quellung method) (The World Health Organisation, 2003). This method was also used to confirm all serotypes determined by PCR. Staff at the Sanger Institute carried out pneumococcal sequencing but I carried out analysis.

2.2 DNA EXTRACTIONS

DNA extractions were carried out using the QIAamp DNA Mini Kit: (51304) by Qiagen (Hilden, Germany) using the published protocol. The only difference being that an in-house bacterial lysis buffer (10mM Tris pH8, 100mM EDTA pH8, 0.5% w/v SDS) was used as an extra step for the lysis of gram-positive bacteria. *H. influenzae* was streaked onto chocolate Colombia blood agar plates, and *S. pneumoniae* was streaked out onto Colombia blood agar plates for 18hrs in an incubator with the conditions of 37°C and 5% (vol/vol) CO_2. 5-10 colonies were picked and placed into 200µl of in-house bacterial lysis buffer, incubated at 37°C for 1 hour. 20µl of Proteinase K was added, mixed by vortexing and incubated at 56°C, 1-3 hours, and vortexed occasionally. The mixture was briefly centrifuged at 6000 rpm in a mini centrifuge to remove condensation droplets from lid. 200µl of Buffer AL was added to the sample, pulse-vortexed for 15s, incubated at 70°C for 10mins and then centrifuged briefly to remove condensation droplets. 200µl of pure ethanol was added, pulse-vortexed for 15s and then was briefly centrifuged to remove condensation droplets from lid. The mixture was applied to a QIAamp Mini spin column without wetting the rim. It was then centrifuged at 8000rpm for 1min. The spin column was then placed in a clean collection tube and filtrate discarded. 500µl of buffer AW1 was added without wetting the rim, and then centrifuged at 8,000rpm for 1min. Spin column was placed in a clean collection tube and filtrate discarded. 500µl

of buffer AW2 was added without wetting the rim, and then centrifuged at 14,000rpm for 3mins. Spin column was placed in a clean collection tube and filtrate discarded. The QIAamp Mini spin column was then placed in a clean 1.5ml micro-centrifuge tube. 200µl of distilled water was added and incubated for 5mins, then centrifuged at 8,000rpm for 1min.

The previous step was repeated and DNA was pooled as a total of 400 µl before being mixed well by pipetting up and down ten times before being re-divided into 2 aliquots of 200 µl of DNA, stored in a-20'C freezer.

2.3 DNA QUANTIFICATION

All quantifications were carried out using the Qubit Fluorometer (Q32857) (Molecular Probes, Invitrogen Carlsbad, California). Reagents were used at room temperature. A working solution was made up by the addition of 1µl of Quant-iT Reagent to 199µl of Quant-iT. This was then incubated at room temperature for 2 minutes. For the standards: 10µl of one standard, (1&2) was added to two 190µl vials of working solution. This resulted in 2 vials of 200µl volumes of standards 1 and 2 which were then incubated at room temperature for 2 minutes. Between 180-199µl of working solution was used to dilute the DNA sample. (Volume must equal 200µl. i.e. 195µl working solution to 5µl DNA sample.) This was then mixed well taking care to avoid air-bubbles. Following calibration using each standard, the concentration of each DNA sample was measured and recorded.

2.4 *H. INFLUENZAE* PCR REACTIONS

2.4.1 *H. INFLUENZAE* SPECIATION AND SEROTYPING PCR PRIMERS

Primers used in the speciation and serotyping of *H. influenzae* were from published sources (See Tables 2.2 and 2.3). Primers were manufactured by Eurogentec, (Custom oligonucleotides, Belgium).

Table 2.2 Speciation and encapsulation primers of *H. influenzae*

Target	Name	Primer sequence (5'-3')	Reference
ompP2	O1	ATA ACA ACG AAG GGA CTA ACG	(Forbes et al., 1992)
ompP2	O3	ACC TAC ACC CAC TGA TTT TTC	(Forbes et al., 1992)
bexB	*BexB*.1F	GGT GAT TAA CGC GTT GCT TAT GCG	(Davis et al., 2011)
bexB	*BexB*.1R	TTG TGC CTG TGC TGG AAG GTT ATG	(Davis et al., 2011)

Table 2.3 Capsule-specific primers of *H. influenzae*

Capsule	Primer	Primer sequence (5'-3')	Reference
a	a1	CTA CTC ATT GCA GCA TTT GC	(Falla et al.,
	a2	GAA TAT GAC CTG ATC TTC TG	(Falla et al.,
	a3	AGT GGA CTA TTC CTG TTA CAC	(Falla et al.,
b	b1	GCG AAA GTG AAC TCT TAT CTC TC	(Falla et al.,
	b2	GCT TAC GCT TCT ATC TCG GTG AA	(Falla et al.,
	b3	ACC ATG AGA AAG TGT TAG CG	(Falla et al.,
c	c1	TCT GTG TAG ATG ATG GTT CA	(Falla et al.,
	c2	CAG AGG CAA GCT ATT AGT GA	(Falla et al.,
	c3	TGG CAG CGT AAA TAT CCT AA	(Falla et al.,
d	d1	TGA TGA CCG ATA CAA CCT GT	(Falla et al.,
	d2	TCC ACT CTT CAA ACC ATT CT	(Falla et al.,
	d3	CTC TTC TTA GTG CTGAAT TA	(Falla et al.,
e	e1	GGT AAC GAA TGT AGT GGT AG	(Falla et al.,
	e2	GCT TTA CTG TAT AAG TCT AG	(Falla et al.,
	e3	CAG CTA TGA ACA AGA TAA CG	(Falla et al.,
	adaptE	TTT GGT AAC GAA TGT AGT GGT AG	HPA
	adaptE	ATA GCT TTA CTG TAT AAG TCT AG	HPA
f	f1	GCT ACT ATC AAG TCC AAA TC	(Falla et al.,
	f2	CGC AAT TAT GGA AGA AAG CT	(Falla et al.,
	f3	AAT GCT GGA GTA TCT GGT TC	(Falla et al.,

Protocols below were adapted for use from that of Falla et al (Falla et al., 1994) and from the Public Health England (PHE) Centre for Infections, Respiratory

and Systemic Infection Laboratory (RSIL) Standard Operating Procedure (personal correspondence with David Litt of the PHE).

2.4.2 *H. INFLUENZAE* OUTER MEMBRANE PROTEIN SPECIATION PCR PROTOCOL

omp primers, O1 and O3 were reconstituted in distilled H_2O to a final concentration of 100 Mm to make up the stock concentration. Working stock was 1:10 dilution (in distilled H2O) of the storage stock. Master and working stocks were stored at -20^{o}C then fully defrosted and kept on ice when being worked with. A master-mix for at least 100 reactions was prepared using the components in table 2.4.

Table 2.4 Reaction mix for *H. influenzae* speciation PCR

Master-mix components	Volumes
Forward Primer	0.625 µl
Reverse Primer	0.625 µl
Biomix Red (Bioline, BIO-25006)	6.25 µl
Distilled H2O	4 µl
DNA sample	1 µl

The master-mix was made up without the addition of DNA, 11.5 µl of the master-mix was aliquoted into 50ul PCR tubes or PCR plates (Sigma-Aldrich) dependent upon sample number. Negative control (dH2O), positive control or sample DNA was added to the mix to give a total of 12.5 µl. The tubes/plates were run in a

Veriti 96-well Thermal Cycler (Applied Biosystems – 4375786) using thermocycling conditions set out in Table 2.5.

Table 2.5 Thermocycling conditions for speciation PCR of *H. influenzae*

Speciation PCR Thermocycling conditions		
95°C for 2 minutes		
25 Cycles of;		
1 minute	94°c	denaturation
1 minute	52°c	annealing
1 minute	72°c	extension
Followed by 1 cycle of 8 minutes at 72°C		

2.4.3 *H. INFLUENZAE* CAPSULATION GENE PCR FOR DETECTION OF THE CAPSULE POLYSACCHARIDE EXPORT INNER-MEMBRANE PROTEIN *BEXB*

The *bexB* capsular gene was amplified using the protocol in 2.4.3. *H. influenzae* OMP speciation PCR protocol, primers and PCR conditions; Table 2.6 and 2.7.

Table 2.6 Reaction mix for the *bexB* encapsulation PCR of *H. influenzae*

Master-mix components	**Volumes**
Forward Primer	1 µl
Reverse Primer	1 µl
Biomix Red (Bioline, BIO-25006)	10 µl
Distilled H2O	6.4 µl
DNA sample	1.6 µl

Table 2.7 Thermocycling conditions for *bexB* encapsulation PCR of *H. influenzae*

Encapsulation PCR Thermocycling conditions		
95°C for 4 minutes		
30 Cycles of;		
30 seconds	95°C	Denaturation
30 seconds	54°C	Annealing
45 seconds	72°C	Extension
Followed by 1 cycle of 8 minutes at 72°C		

2.4.4 *H. INFLUENZAE* SEROTYPING PCR

H. influenzae capsular genes were amplified using the protocol in 2.4.3. *H. influenzae* OMP speciation PCR protocol, with the primers and conditions listed in tables 2.8 to 2.10.

Table 2.8 Capsule specific primer pairs for serotyping of *H. influenzae*

Capsule specific forward primer	**Capsule specific reverse primer**
a1	a2
b1	b2
c1	c2
d1	d2
Adapted e1	Adapted e2
f1	f3

Table 2.9 Reaction mix for the serotyping PCR of *H. influenzae*

Master-mix components	**Volumes**
Forward Primer	0.625ul
Reverse Primer	0.625ul
Biomix Red (Bioline, BIO-25006)	6.25ul
Distilled H2O	4ul
DNA sample	1ul

Table 2.10 Thermocycling conditions for the serotyping of *H. influenzae*

Serotyping PCR Thermocycling conditions		
95°C for 2 minutes		
25 Cycles of;		
60 seconds	94°C	Denaturation
60 seconds	52°C	Annealing
60 seconds	72°C	Extension
Followed by 1 cycle of 8 minutes at 72°C		

2.4.5 CONTROL STRAINS OF *H.INFLUENZAE*

The control strains used in all PCR reaction sets are listed in Table 2.11 and were provided by the staff of the PHE, RSIL. See http://www.lgcstandards-atcc.org/ for details of these strains.

Table 2.11 *H. influenzae* species control strains

Species	**Strain Name**
H. influenzae serotype *b*	ATCC 10211
H. influenzae serotype *b*-	
H .influenzae non-capsulated	ATCC 49247
H. parainfluenzae	NCTC 7857
H. influenzae serotype a	ATCC 9006
H. influenzae serotype c	ATCC 9007
H. influenzae serotype d	ATCC 9008
H. influenzae serotype e	ATCC 8142
H. influenzae serotype f	ATCC 9833

Table 2.12 Expected PCR product size for *H. influenzae* serotyping PCR

Primer pair	**Expected product**
a1/a2	250bp
b1/b2	480bp
c1/c2	250bp
d1/d2	150bp
e1/e2	1360bp
f1/f3	345bp
Omp	1000bp
BexB	567bp

2.5 MULTI-LOCUS SEQUENCE TYPING (MLST)

2.5.1 *H. INFLUENZAE* MLST

Bacterial DNA was extracted from each isolate using the QIAGEN protocol outlined in section 2.2. Multi locus sequence typing (MLST) (Enright and Spratt, 1998) was carried out by QIAGEN Genomic services (Germany). The method is well documented (Spratt, 1999, Enright et al., 2000); briefly seven sets of primers for seven housekeeping genes of *H. influenzae* were used to amplify an internal fragment for each gene, which remains conserved in all *H. influenzae* organisms thus allowing identification. Allelic profiles were assigned on genetic difference with unique sequence type (ST) assigned as overall indicator of the strain profile (Meats et al., 2003a).

Table 2.13 *H. influenzae* MLST primers (Meats et al., 2003a)

Housekeeping gene	Sequence
adk-up	5'-GGTGCACCGGGTGCAGGTAA-3
adk-dn	5'-CCTAAGATTTTATCTAACTC-3'
atpG-up	5'-ATGGCAGGTGCAAAAGAGAT-3'
atpG-dn	5'-TTGTACAACAGGCTTTTGCG-3'
frdB-up	5'-CTTATCGTTGGTCTTGCCGT-3'
frdB-dn	, 5'-TTGGCACTTTCCACTTTTCC-3'
fucK-up	5'-ACCACTTTCGGCGTGGATGG-3'
fucK-dn	5'-AAGATTTCCCAGGTGCCAGA-3'
mdh-up	5'-TCATTGTATGATATTGCCCC-3'
mdh-dn	5'-ACTTCTGTACCTGCATTTTG-3'
pgi-up	5'-GGTGAAAAAATCAATCGTAC-3'
pgi-dn	5'-ATTGAAAGACCAATAGCTGA-3'
recA-up	5'-ATGGCAACTCAAGAAGAAAA-3'
recA-dn	5'-TTACCAAACATCACGCCTAT-3'

2.5.2 MLST ANALYSIS

eBurst v2 was used to identify relationships between the study strains and those previously deposited in the *H. influenzae* MLST database (Feil et al., 2004) (eBurst Software - http://eburst.mlst.net/default_v2.asp). eBurst is able to indicate

the evolutionary relationships between MLST types with the purpose of highlighting a predicted founder genotype (Feil et al., 2004).

2.6 DIAGNOSTIC TESTING TO DETERMINE PNEUMOCOCCAL TYPES

2.6.1 MULTIPLEX PCR TO DEDUCE PNEUMOCOCCAL SEROTYPES

PCR was performed on pneumococcal isolates using primer combinations recommended by the CDC **(**http://www.cdc.gov/ncidod/biotech/strep/pcr.htm, http://www.cdc.gov/ncidod/biotech/files/pcr-US-clinical-specimens-March2010.pdf)

Table 2.14 Reaction mix for typing pneumococcal isolates in 12.5 ul volume

Concentration	Reaction 1	(µL)/rxt
n/a	PCR H2O	Varies
50mM	$MgCl_2$	**0.5**
100µM	Fwd Primers	Varies
100µM	Rev Primers	Varies
2x	Red PCR Mix	**6.25**
n/a	Crude DNA	**1**
	Total	**12.5**

Table 2.15 Multiplex PCR pools for deducing serotypes of pneumococcal isolates

Primer Info*		**To get final concentration:**	**Conc. (µM) 1 rxn**
Reaction 1	**Original Conc. (µM)**	**Primer (µL)**	
CPSA-F (913424)	100	0.0125	0.1
CPSA-R (913425)	100	0.0125	0.1
6(6A/6B/6C)-F (914837)	100	0.0375	0.3
6(6A/6B/6C)-F (914838)	100	0.0375	0.3
3-F (914831)	100	0.0375	0.3
3-R (914832)	100	0.0375	0.3
19A-F (914855)	100	0.0375	0.3
19A-F (914856)	100	0.0375	0.3
22F/22A-F (903763)	100	0.0625	0.5
22F/22A-F (903764)	100	0.0625	0.5
16F-F (918138)	100	0.05	0.4
16F-F (918139)	100	0.05	0.4
Primer Info*		**To get final concentration:**	**Conc. (µM) 1 rxn**
Reaction 2	**Original Conc. (µM)**	**Primer (µL)**	
CPSA-F (913424)	100	0.0125	0.1
CPSA-R (913425)	100	0.0125	0.1
8-F (911983)	100	0.025	0.2
8-R (911984)	100	0.025	0.2
33F/33A/37-F (807842)	100	0.0375	0.3
33F/33A/37-R (807843)	100	0.0375	0.3
15A/15F-F	100	0.0375	0.3
15A/15F-R	100	0.0375	0.3
7F/7A-F	100	0.05	0.4
7F/7A-R	100	0.05	0.4
23A-F	100	0.0625	0.5
23A-R	100	0.0625	0.5
Primer Info*		**To get final concentration:**	**Conc. (µM) 1 rxn**
Reaction 3	**Original Conc. (µM)**	**Primer (µL)**	
CPSA-F	100	0.0125	0.1
CPSA-R	100	0.0125	0.1
19F-F	100	0.0625	0.5
19F-R	100	0.0625	0.5
12F/12A/44/46-F	100	0.0625	0.5
12F/12A/44/46-R	100	0.0625	0.5

11A/11D-F	100	0.0375	0.3
11A/11D-R	100	0.0375	0.3
38/25F-F	100	0.0375	0.3
38/25F-R	100	0.0375	0.3
35B-F	100	0.0625	0.5
35B-R	100	0.0625	0.5
Primer Info*		**To get final concentration:**	**Conc. (µM) 1 rxn**
Reaction 4	**Original Conc. (µM)**	**Primer (µL)**	
24(24A/24B/24F)-F	100	0.0125	0.1
24(24A/24B/24F)-R	100	0.0125	0.1
CPSA-F	100	0.0125	0.1
CPSA-R	100	0.0125	0.1
7C/7B/40-F	100	0.0375	0.3
7C/7B/40-R	100	0.0375	0.3
4-F	100	0.0375	0.3
4-R	100	0.0375	0.3
18(18A/18B/18/C/18F)-F	100	0.0375	0.3
18(18A/18B/18/C/18F)-R	100	0.0375	0.3
9V/9A-F	100	0.0625	0.5
9V/9A-R	100	0.0625	0.5
Primer Info*		**To get final concentration:**	**Conc. (µM) 1 rxn**
Reaction 5	**Original Conc. (µM)**	**Primer (µL)**	
CPSA-F	100	0.0125	0.1
CPSA-R	100	0.0125	0.1
14-F	100	0.0375	0.3
14-R	100	0.0375	0.3
I-F	100	0.0375	0.3
I-R	100	0.0375	0.3
23F-F	100	0.0625	0.5
23F-R	100	0.0625	0.5
15B/15C-F	100	0.0375	0.3
15B/15C-R	100	0.0375	0.3
10A-F	100	0.0625	0.5
10A-R	100	0.0625	0.5
Primer Info*		**To get final concentration:**	**Conc. (µM) 1 rxn**
Reaction 6	**Original Conc. (µM)**	**Primer (µL)**	
39-F	100	0.025	0.2

39-R	100	0.025	0.2
CPSA-F	100	0.0125	0.1
CPSA-R	100	0.0125	0.1
10F/10C/33C-F	100	0.0375	0.3
10F/10C/33C-R	100	0.0375	0.3
5-F	100	0.0375	0.3
5-R	100	0.0375	0.3
35F/47F-F	100	0.0375	0.3
35F/47F-r	100	0.0375	0.3
17F-F	100	0.0625	0.5
17F-R	100	0.0625	0.5
Primer Info*		**To get final concentration:**	**Conc. (µM) 1 rxn**
Reaction 7	**Original Conc. (µM)**	**Primer (µL)**	
CPSA-F	100	0.0125	0.1
CPSA-R	100	0.0125	0.1
23B-F	100	0.025	0.2
23B-R	100	0.025	0.2
35A/35C/42-F	100	0.0375	0.3
35A/35C/42-R	100	0.0375	0.3
34-F	100	0.0375	0.3
34-R	100	0.0375	0.3
9N/9L-F	100	0.0625	0.5
9N/9L-R	100	0.0625	0.5
31-F	100	0.0625	0.5
31-R	100	0.0625	0.5
Primer Info*		**To get final concentration:**	**Conc. (µM) 1 rxn**
Reaction 8	**Original Conc. (µM)**	**Primer (µL)**	
CPSA-F	100	0.0125	0.1
CPSA-R	100	0.0125	0.1
21-F	100	0.025	0.2
21-R	100	0.025	0.2
2-F	100	0.0375	0.3
2-R	100	0.0375	0.3
20-F	100	0.0375	0.3
20-R	100	0.0375	0.3
13-F	100	0.05	0.4
13-R	100	0.05	0.4
Primer Info*		**To get final concentration:**	**Conc. (µM) 1 rxn**
Reaction 6C	**Original Conc. (µM)**	**Primer (µL)**	

CPSA-F	100	0.0125	0.1
CPSA-R	100	0.0125	0.1
6A/6B/6C-F	100	0.0375	0.3
6A/6B/6C-R	100	0.0375	0.3
6C-F	100	0.0625	0.5
6C-R	100	0.0625	0.5

2.6.2 SLIDE AGGLUTINATION TO CONFIRM PNEUMOCOCCAL SEROTYPES

Preparation of pneumococcal isolates:

Using a sterile loop and aseptic technique, one bead was removed from the cryovial and streaked onto a fresh agar plate (Columbia blood agar or BHI agar). Plates were incubated upside down at 35°C in 4% CO_2 incubator for 15-18 hours (overnight). Using a sterile loop and aseptic technique an isolated colony or sweep of colonies was removed from the plate and transferred to 5ml of liquid media (Todd Hewitt Broth) in a screw-cap universal. Cultures were incubated with loose lids at 35oC in 4%CO2 incubator for 15-18 hours (overnight). Overnight cultures were spun down at 1500rpm for 30 minutes and supernatant removed leaving 200ul and pellet, pellets were re-suspended by vortexing or pipetting mixing.

Slide Agglutination:

The suspension was used to perform slide agglutination using appropriate Statens Serum Institute antisera or latex factors raised against pneumococcal polysaccharide capsular antigens. 10**µ**l of culture was dropped on a glass slide using a pipette or 10ul sterile loop. 10**µ**l of Antisera or 1**µ**l of latex factor was added and the slide rocked to mix reagent and culture. Agglutination (clumping with clearing background) of the suspension was recorded as a positive result. If no agglutination

was seen, gentle rocking of the slide for a few seconds (up to 30 seconds) was performed to observe a positive result. If there was no agglutination on repeat testing, the test was repeated with pneumococcal 10**µ**l omniserum (polyvalent antiserum containing antibodies to all 90 known serotypes of pneumococcal capsular polysaccharide). If there was agglutination with omniserum original PCR results were again checked and after investigation if still not satisfactory, isolates were sent to Staten Serum Institute (SSI). If there is no agglutination with omniserum isolates were treated as possible non-pneumococcus and sent to SSI to confirm.

Statens Serum Institute confirmation of pneumococcal serotypes:

Isolates that could not be assigned a serotype through multiplex PCR or in-house slide agglutination were sent to SSI as bacterial cultures suspended in glycerol for confirmation Quellung testing. Any isolate that produced mis-matched results between the different methods of typing were excluded from the analysis in this thesis until further investigations could be performed.

2.7 WHOLE GENOME SEQUENCING

2.7.1 SEQUENCING PERFORMED AT THE SANGER INSTITUTE

Isolates of *S. pneumoniae* were cultured and had DNA extracted as per Chapter 2.2 and 2.3 but eluted into 60**µ**l TE buffer instead of 200**µ**l. 50**µ**l of DNA was added to a 96 deep-well PCR plate and packaged in dry ice before being delivered to the Sanger Institute via an overnight courier. Isolates were then processed and sequenced at the Sanger Institute using the Illumina HiSeq 2500 platform and results were received via FTP transfer upon completion.

2.7.2 SEQUENCING PERFORMED IN-HOUSE

Isolates of *H. influenzae* were sequenced in house by two technicians of our group and myself due to the volume of isolates that required sequencing.

Protocol for Illumina Miseq whole genome sequencing:

Nextera Kit: (FC-121-1031 – Illumina)

(A) Tagmentation of Genomic DNA:

During this step genomic DNA was tagmented (tagged and fragmented) by the Nextera transposome.

Table 2.16 Nextera Tagmentation consumables

Item	Quantity	Storage	Supplied by
TD Tagment DNA buffer	1 tube	-15 to -25°C	Illumina
TDE1 Tagment DNA enzyme	1 tube	-15 to -25°C	Illumina
Genomic DNA (2.5ng/ul)	# tubes	-15 to -25°C	User

In a new 0.2 ml PCR tube (A) 20µl of genomic DNA at 2.5 ng/µl (50 ng total) was added, 25µl of TD Buffer was added to the tube containing genomic DNA. 5µl of TDE1 was added to the tube containing genomic DNA and TD Buffer. Tube (A) was mixed and centrifuged at 280 xg at 20 ºC for 1 minute.Tube (A) was placed in a thermocycler with the following program:

- 55 ºC for 5 minutes
- Hold at 10 ºC

(B) Clean-Up of Tagmented DNA:

Table 2.17 Nextera DNA clean-up consumables

Item	Quantity	Storage	Supplied by
RSB (Resuspension Buffer)	1 tube	-15° to -25°C	Illumina
Zymo DNA binding buffer	1 bottle	Room temperature	Zymo
Zymo wash buffer 1 bottle Room temperature Zymo	1 bottle	Room temperature	Zymo
Zymo spin columns			Zymo

The tagmented DNA was purified from the Nextera transposome.

Zymo clean-up: (D4011 – Zymo)

In a new 0.5 ml tube (B), 50µl of Zymo DNA binding buffer was added. 50µl from tube (A) was transferred to the new tube (B) containing Zymo DNA binding buffer and was mixed gently. The spin column was placed into a 2 ml collection tube (provided) on the rack. Sample mixture from the tube (B) was transferred to the corresponding spin column. The spin column was centrifuged at 1,300 xg at 20°C for 2 minutes. Flow-through was discarded.

Zymo spin column wash:

a) 300µl of wash buffer was added to each spin column.

b) Centrifuged at 1,300 xg at 20°C for 2 minutes. Flow-through was discarded.

Above step was repeated for a total of 2 washes; the spin column was then centrifuged at 1,300 xg for 2 minutes to ensure no residual wash buffer was present. Spin column was placed into a new 1.5ml tube (C). 25µl of RSB was added directly to the spin column. This was then incubated for 2 minutes at room temperature. Tube (C) was then centrifuged at 1,300 xg at 20°C for 2 minutes. Flow through was kept.

(C) PCR Amplification: Nextera 24 samples index primer kit: (FC-121-1011 – Illumina)

In this step, the purified tagmented DNA was amplified via a limited-cycle PCR program.

Table 2.18 Nextera PCR consumables

Item Quantity Storage Supplied By	Quantity	Storage	Supplied by
NPM (Nextera PCR Master Mix)	1 tube	-15° to -25°C	Illumina
PPC (PCR Primer Cocktail)	1 tube	-15° to -25°C	Illumina
Index 1 primers (N7XX)	1 tube	-15° to -25°C	Illumina
Index 2 primers (N5XX)	1 tube	-15° to -25°C	Illumina

Table 2.19 Nextera index primer matches

(1)	(2)	N501	N502	N503	N504
N701		S(1): N501+N701	S (2): N502+N701	S (3): N503+N701	S (4): N504+N701
N702		S (5): N501+N702	S (6): N502+N702	S (7): N503+N702	S (8): N504+N702
N703		S (9): N501+N703	S (10): N502+N703	S (11): N503+N703	S (12): N504+N703
N704		S (13): N501+N704	S (14): N502+N704	S (15): N503+N704	S (16): N504+N704
N705		S (17): N501+N705	S (18): N502+N705	S (19): N503+N705	S (20): N504+N705
N706		S (21): N501+N706	S (22): N502+N706	S (23): N503+N706	S (24): N504+N706

In a new 0.2ml PCR tube (D), 5 µl of index 2 primers (white caps) was added. 5 µl of index 1 primers (orange caps) was added to each tube (D). 15 µl of NPM was added to tube (D) containing index primers. Add 5 µl PPC was added to tube (D) containing index primers and NPM. 20 µl of purified tagmented DNA was transferred from the tube (C) to the corresponding tube (D). This was then mixed and centrifuged briefly.

The below PCR settings were then used:

• 72°C for 3 minutes

• 98°C for 30 seconds

• 5 cycles of: 98°C for 10 seconds/63°C for 30 seconds/72°C for 3 minutes

• Hold at 10°C

(D) PCR Clean-UP: (A63880 – Agencourt)

Procedure:

Tube (D) was centrifuged at 280 xg for 1 min (20˚C) to collect condensation. In a new 1.5ml tube (E), 50 µl of the PCR product was transferred from the tube (D). AMPure XP beads were vortexed for 30 seconds. 30 µl of AMPure XP beads were added to tube (E) and mixed. This was then incubated at room temperature without shaking for 5 minutes. Tube (E) was placed on a magnetic stand for 2 minutes. With the tube (E) on the magnetic stand, the supernatant was carefully removed and discarded using a pipette. With the tube (E) on the magnetic stand, beads were washed twice with freshly prepared 80% ethanol. Incubate the tube (E) on the magnetic stand for 30 seconds or until the supernatant appears clear. The supernatant was then removed and discarded. With the tube (E) still on the magnetic stand, the beads were allowed to air-dry for 15 minutes. Tube (E) was removed from the magnetic stand. 32.5 µl of RSB was added to tube (E). This was mixed and incubated at room temperature for 2 minutes. Tube (E) was placed on the magnetic stand for 2 minutes. New tube (F) was labelled. 30 µl of the supernatant from the tube (E) was transferred to the tube (F). Tube (F) was be stored at -20ºC.

(E) Pool Libraries:

Procedure:

10µl of sample library was transferred from tube (F) to a new 0.2ml PCR tube (G). The concentration of sample library in each tube (G) was normalized to 4nM using “Tris-Cl 10mM, pH 8.5 with 0.1% Tween 20”. Tube (G) was then mixed and centrifuged In a new 0.5/ 1.5ml tube (H), 5µl of each sample of tube (G) was transferred. This was mixed gently. It was stored at -20 until sequencing

2.8 16S DNA COMMUNITY PROFILING PROTOCOL

2.8.1 SAMPLE PREPARATION

Samples were prepared from a variety of starting materials; they were either subjected to media enrichment or direct lysis for DNA extraction. Samples used in bacterial community analysis were: glycerol stocks, bead stocks or nasopharyngeal swab heads that had been stored in frozen glycerol (see section 2.1.6). From the bead stock, 3 beads were removed from a slightly thawed cryotube and either 100ul of glycerol or a whole swab head were removed aseptically from thawed sample tubes.

2.8.2 SAMPLE ENRICHMENT PROTOCOL

The sample to be enriched was added to 1.5mls of enriched BHI (5ml BHI, 50ul Hemin, 1ul B-NAD) in a sterile centrifuge tube. The sample was then incubated without shaking at 37°C at 5% CO2 for approximately 4 to 5 hours with lids lightly taped to allow air circulation whilst reducing the potential of a liquid spill. Bacteria were then pelleted by centrifugation for 10 min at 5000 x g (7500 rpm). Bacterial pellets were suspended in 180µl of in-house bacterial lysis buffer prior to DNA isolation as described in section 2.2.

2.8.3 DIRECT LYSIS METHOD

500µl of in-house bacterial lysis buffer (see section 2.2) was added and incubated at 37°C with 5% CO2 for 1 hour. DNA isolation protocol 2.2, step 2 was then followed.

2.9 16S DNA COMMUNITY PROFILING – SEQUENCING PROTOCOL

2.9.1 16S AMPLIFICATION PRIMER PROTOCOL

Primers were made up to given concentrations, as specified by content, in distilled H_2O (100mM). They were further diluted 1:10 for working primer mix (10mM).

Once all components were thawed and vortexed, individual components were worked with on ice.

Table 2.20 Samples and corresponding barcoded primers for 16S amplification

Sample	Primer and barcode name	Sequence of primer, linker and barcode

Water	16S-27F_MID4	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGAGAGTTTGATCCTGGCTCAG
Positive swab	16S-27F_MID6	CCATCTCATCCCTGCGTGTCTCCGACTCAGATATCGCGAGAGAGTTTGATCCTGGCTCAG
Negative swab	16S-27F_MID7	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTAAGAGTTTGATCCTGGCTCAG
3189 bead	16S-27F_MID8	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCGTGTCAGAGTTTGATCCTGGCTCAG
3294 bead	16S-27F_MID10	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGAGAGTTTGATCCTGGCTCAG
4131 bead	16S-27F_MID11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTAGAGTTTGATCCTGGCTCAG
5015 swab head	16S-27F_MID14	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGAGATACAGAGTTTGATCCTGGCTCAG
5027 glycerol	16S-27F_MID15	CCATCTCATCCCTGCGTGTCTCCGACTCAGATACGACGTAAGAGTTTGATCCTGGCTCAG
5027 swab	16S-27F_MID16	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGTACTAAGAGTTTGATCCTGGCTCAG
5027 swab vortexed	16S-27F_MID17	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTCTAGTACAGAGTTTGATCCTGGCTCAG
ALL	16S-338Rev_Pri	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCTGCTGCCTCCCGTAGGAGT

Master-mix was made up per primer set for the given number of reactions, master mix term excluded DNA. Twenty two µl of master mix was added to each reaction well; the DNA was then added and pipetted up and down gently to mix, on ice.

Table 2.21 16S amplification reaction mix

16S PCR reaction mix

1ul forward primer
1ul reverse primer
14ul Bioline red mix
4ul H_2O
2ul $MgCl_2$
3ul DNA

The plate/individual PCR tubes were sealed before being placed in the thermo-cycler under the correct conditions.

Table 2.22 16S PCR Thermo-cycling conditions

Thermocycling conditions for 16S PCR	
95°C for 4 minutes	
35 Cycles of;	
30 seconds	94°C
60 seconds	51°C
90 seconds	72°C
Followed by 1 cycle of 10 minutes at 72°C	

2.9.2 POST 16S PCR AMPLIFICATION CLEAN-UP PROTOCOL

The QIAQuick Kit: (28106 – Qiagen) was used to clean PCR products following amplification. The protocol was taken from official handbook. Five volumes

of Buffer PB were added to one volume of the PCR sample and mixed. If the color of the mixture was orange or violet, 10µl of 3 M sodium acetate – pH 5.0 was added then mixed. A QIAquick spin column was placed in a provided 2 ml collection tube. The sample was applied to the QIAquick column and centrifuged for 30–60 s. The flow-through was discarded. The QIAquick column was placed back into the same tube. 0.75 ml Buffer PE was added as a wash to the QIAquick column and centrifuged for 30–60 s. Flow-through was discarded and the QIAquick column was placed back in the same tube and the column was centrifuged for an additional 1 min. QIAquick column was placed in a clean 1.5 ml micro centrifuge tube. For the elution of the DNA, 50µl of Buffer EB (10mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) was added to the center of the QIAquick membrane and centrifuged for 1 min. Alternatively, for increased DNA concentration, 30µl of elution buffer was added to the center of the QIAquick membrane, the column was incubated for a further one minute, before being centrifuged.

2.9.3 AMPLICON QUALITY AND QUANTIFICATION

Purified DNA was viewed on a 2% (w/v) agarose TAE (TAE - 10 mM Tris-Cl, pH 7.5

1 mM EDTA) gel. One volume of loading dye (Bioline Reagents, UK) was added to 5 volumes of purified DNA. The solution was mixed by pipetting up and down before loading approximately 10µl into each well. The gel was run at 180 volts for 90 minutes to separate bands. The reaction mix was run with loading dye on a gel along with a 1Kb gel marker (Bioline Reagents, UK) in a separate well, using Gel Red (Bioscience, UK) as an in-gel stain. Band size and quality was determined visually using UV light.

2.9.4 ROCHE GS JUNIOR SEQUENCING RATIONALE

Samples were sent to the PHE Health Protection Services, Colindale, on dry ice, by same day courier for sequencing of the 16S gene on a Roche GS Junior sequencer. Results were received in the format of a .sff file. These files were submitted to the online website, MG-RAST (Meyer et al., 2008) for assignment to a database of bacterial sequences to genus level.

2.10 BIOINFORMATICS ANALYSIS

2.10.1 QUALITY CONTROL

Fastq sequences were analysed using a program called FastQC, (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Isolates with less than 20 times coverage were reported for repeat sequencing.

2.10.2 GENOME ASSEMBLY

Genome assembly was carried out using a program called Masurca, (http://www.genome.umd.edu/masurca.html/).

2.10.3 *IN SILICO* PCR

In silico PCR was performed using a program called IPCRESS, (http://manpages.ubuntu.com/manpages/precise/man1/ipcress.1.html), with the sequences of the primers used for wet-lab PCR for both *S. pneumoniae* and *H. influenzae.*

2.10.4 *IN SILICO* MLST

In silico MLST typing was carried out using a program called SRST2, (http://katholt.github.io/srst2/)(Inouye et al., 2012) (Inouye et al., 2014), using the paired raw and trimmed reads of the fastq.gz sequence files of the isolates.

2.10.5 *IN SILICO* VIRULENCE PROFILLING

In silico virulence factor typing was carried out using SRST2.

2.10.6 *IN SILICO* ANTIBIOTIC RESISTANCE PROFILLING

In silico antibiotic resistance typing was carried out using SRST2.

2.11 STATISTICAL ANALYSIS

95% confidence intervals were calculated for all graphs presented in this thesis. Confidence intervals provide a range of values that estimates the population value to a confidence level of 95%.

2.12 DATA-SET USED FOR ANALYSIS

Full details of the data set used for the analysis in this thesis is listed in Appendix 1

3. CARRIAGE OF RESPIRATORY PATHOGENS IN THE NASOPHARYNX OF YOUNG CHILDREN DURING THE IMPLEMENTATION OF PCVS IN THE UK

3.1 BACKGROUND

PCV7 was added to the UK NIP in 2006 and resulted in a decrease of VT serotypes. Following serotype replacement and an increase in disease caused by NVTs, PCV13 was added to the UK NIP in 2010 and led to a further reduction in IPD due to the additional serotypes covered by the vaccine.

PCV7 has already been associated with changes in the populations of other nasopharyngeal niche species including *H. influenzae* and *S. aureus* (van Gils et al., 2011a, Spijkerman et al., 2012, Dunne et al., 2013); this may have implications in terms of species replacement in carriage and therefore disease (Wiertsema et al., 2011). Since the introduction of PCV13 in the UK in 2010, microbial population dynamics in the nasopharynx of UK children may have undergone further change. It has been proposed that due to high case: carrier ratios (CCRs) and low carriage of some PCV10/13 serotypes that there would be a low chance of serotype replacement when the new vaccines were implemented but that carriage should be monitored as some serotypes with high CCRs could reduce the benefits of PCV vaccination (Flasche et al., 2011). The data presented in this chapter aims to inform future vaccine needs by indicating if serotype replacement such as that observed after the introduction of PCV7 (Hicks et al., 2007, Weinberger et al., 2011, Miller et al., 2011) is accompanied by a change in the microbial community of the nasopharynx post PCV13 implementation.

3.2 OBJECTIVES

- To determine the carriage rates of *S. pneumoniae*, *H. influenzae*, *S. aureus*, *M. catarrhalis* and *N. meningitidis* in children aged 4 years and under.
- To compare carriage rates of the included bacterial species at each time-point of the study. Study time points ran each year from October to the following March beginning 2006/07 and ending 2012/13.
- To compare carriage rates of the included bacterial species across 3 time-points related to PCV-implementation. The PCV7 era, 2006/07 and 2007/08, the pre PCV13/post PCV7 era, 2008/09 and 2009/10 and the post PCV13 era, 2010/11, 2011/12 and 2012/13.

3.3 RESULTS

3.3.1 CARRIAGE OF SELECTED BACTERIAL SPECIES

Trends in carriage rates over the 6-year period were analysed by comparing the numbers of swabs that cultured a bacterial species each year. The rate of co-carriage between *S. pneumoniae* and *H. influenzae* was estimated by recording each occasion they were both cultured together from a single swab.

The percentage of swabs colonised with *S. pneumoniae* was 32.1% (104) during 2006/07 and 27.9% (104) during 2007/08. However during 2008/09 when detection of four additional bacterial species was included (Table 3.1), the percentage of swabs from which any selected bacterial species was cultured was 47.6%. The study expanded in 2009/10 to include *M. catarrhalis* as well as the species of the previous year and the percentage of swabs that cultured any of the six species rose to 68.2%. The bacteria included for detection remained the same from 2009/10 over 4-study time points to 2012/13.

PCV13 was added to the UK paediatric NIP in April 2010 ahead of the sampling period for 5th time-point of the carriage study, ran from October to March 2010/11. By the 6th year (October to March 2011/12), a decrease of 4.5% from 74.2% (95% CI: 68.7 - 78.9) to 69.7% (95% CI: 64.5 - 74.4) in the prevalence of colonised swabs was observed (figure 3.1); this decrease is interesting but not significant at the 95% level (p=>0.05).

Table 3.1: Carriage rate of bacterial isolates and percentage of bacterial species selected for study

Year		*SP*	*HI*	*NM*	MSSA	MRSA	*MC*
2006/07	**Number**	**104**					
	%	32.1					
	95% CI	27.0 - 37.2					
2007/08	**Number**	**104**					
	%	27.9					
	95% CI	23.4 - 32.5					
2008/09	**Number**	**102**	**60**	**0**	**12**	**0**	
	%	31.1	18.3	0.0	3.7	0.0	
	95% CI	26.1 - 36.1	14.1 - 22.5	-	1.7 - 5.7	-	
2009/10	**Number**	**111**	**70**	**0**	**41**	**1**	**116**
	%	27.8	17.5	0.0	10.3	0.3	29.1
	95% CI	23.4 - 32.2	13.8 - 21.2	-	8.0 - 12.6	(-)0.2 - 0.8	24.6 - 33.6
2010/11	**Number**	**107**	**69**	**0**	**17**	**1**	**95**
	%	37.3	24.0	0.0	5.9	0.3	33.1
	95% CI	31.7 - 42.9	19.1 - 28.9	-	3.2 - 8.6	(-)0.3 - 0.9	27.7 - 38.5
2011/12	**Number**	**105**	**62**	**2**	**15**	**0**	**105**
	%	31.5	18.6	0.6	4.5	0.0	31.5
	95% CI	26.5 - 36.5	14.8 - 26.4	(-)0.2 - 1.4	2.3 - 6.7	-	26.5 - 36.5
2012/13	**Number**	**77**	**51**	**0**	**10**	**0**	**68**
	%	34.5	22.9	0.0	4.5	0.0	30.5
	95% CI	28.3 - 40.7	17.4 - 28.4	-	1.8 - 7.2	-	24.5 - 36.5

SP = *S. pneumoniae*, HI = *H. influenzae,* NM = *N. meningitidis*, MS/MRSA = Methicillin sensitive/Methicillin resistant *S. aureus*, MC = *M. catarrhalis*

FIGURE 3.1: PAEDIATRIC NASOPHARYNGEAL SWABS COLONISED WITH AT LEAST ONE SPECIES

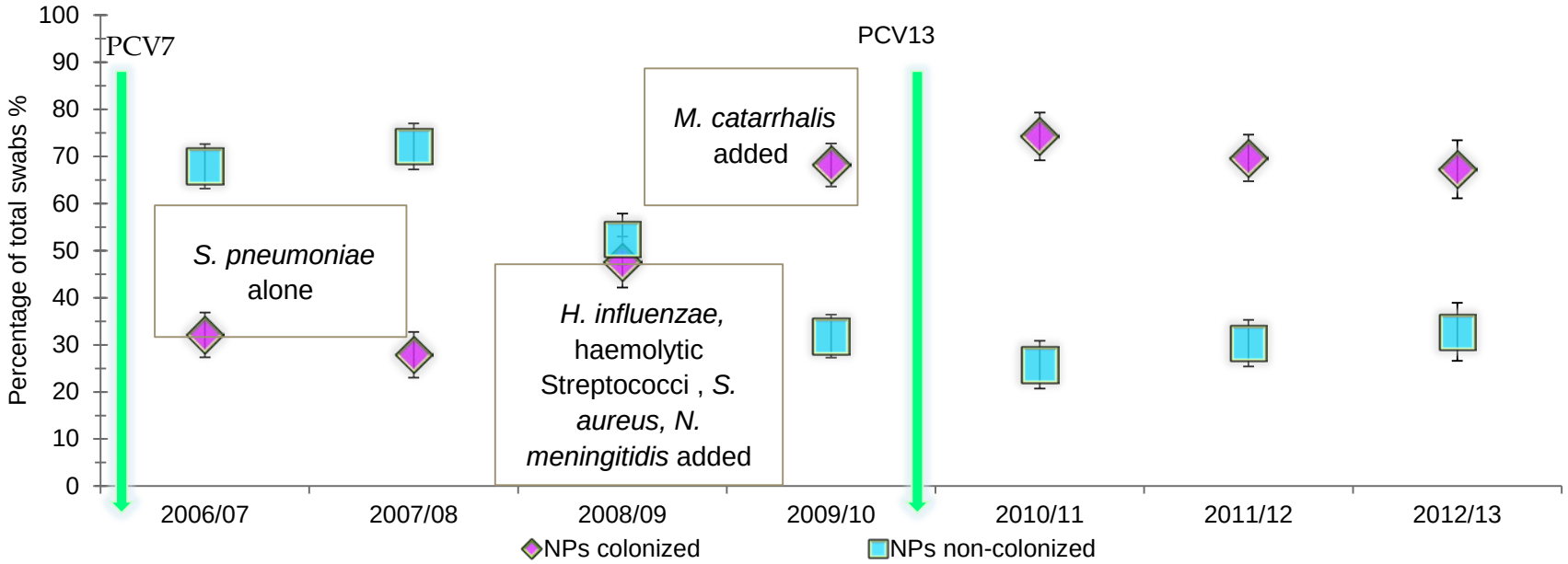


Figure showing the percentage of swabs found to be colonised with at least one species of bacteria at each time-point of the study, from 2006/07 to 2012/13. Purple markers represent the percentage of swabs colonised with at least one species of bacteria and blue markers represent the percentage of swabs not colonised with any species of bacteria.

3.3.2 CARRIAGE OF SPECIFIC BACTERIAL SPECIES OVER THE SEVEN-YEAR STUDY PERIOD (2006/07 TO 2012/13)

Figure 3.2 shows the percentage of each species found to colonise the nasopharyngeal swabs of the sample group. The percentage shown was derived from the numbers of each species detected from the total of all swabs taken per study year. *S. pneumoniae* was the most frequently detected species during 2008/09, when *H. influenzae*, *N. meningitidis* and *S. aureus* were first included in the study. When *M. catarrhalis* was included in years 2009/10 to 2012/13 of the carriage study, it and *S. pneumoniae*, were the most frequently detected species. *H. influenzae* was the third most detected species after *S. pneumoniae* and *M. catarrhalis* in years 2009/10 to 2012/13. These observations provide an overview of the abundance of clinically relevant species contributing to the nasopharyngeal microbiota as detected by culture and how changes in carriage can change the observed bacterial hierarchy in the nasopharynx of children.

3.3.3 TRENDS IN COLONISATION OVER TIME

No significant change in the detection of microbial carriage of selected organisms was observed, with the exception of MSSA. MSSA increased from 12 isolates (3.7%, 95% CI: 1.7% - 5.7%) in 2008/09 to 41 isolates (10.3%, 95% CI: 8.0% - 12.6%) in 2009/10, before decreasing steadily to 15 and 10 isolates (4.5%) in both 2011/12 and 2012/13.

The data set was split into three categories surrounding PCV implementation into the UK National Immunisation Program. These time divisions allow a snapshot of the bacterial carriage observed when vaccine implementation is accounted for. Study

years 2006/07 to 2007/08 was categorized as the PCV7 era, study years 2008/09 to 2009/10 was categorized as the post-PCV7/pre-PCV13 era and study years 2010/11, 2011/12 and 2012/13 was categorized as the PCV13 era. Figure 3.2b highlights that between the PCV7 era and post-PCV7/pre-PCV13 era there was no changes in carriage for *S. pneumoniae*. For *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* there were no significant changes in the PCV13 era for the above species compared to the post-PCV7/pre-PCV13 era. For MSSA there was a non-significant decrease observed in the PCV13 era compared to the post-PCV7/pre-PCV13 era. *N. meningitidis* and MRSA were detected too infrequently to indicate any changes between vaccine eras in this study.

FIGURE 3.2A: PAEDIATRIC NASOPHARYNGEAL SWABS COLONISED WITH SPECIFIC BACTERIAL SPECIES (2006/07 – 2012/13)

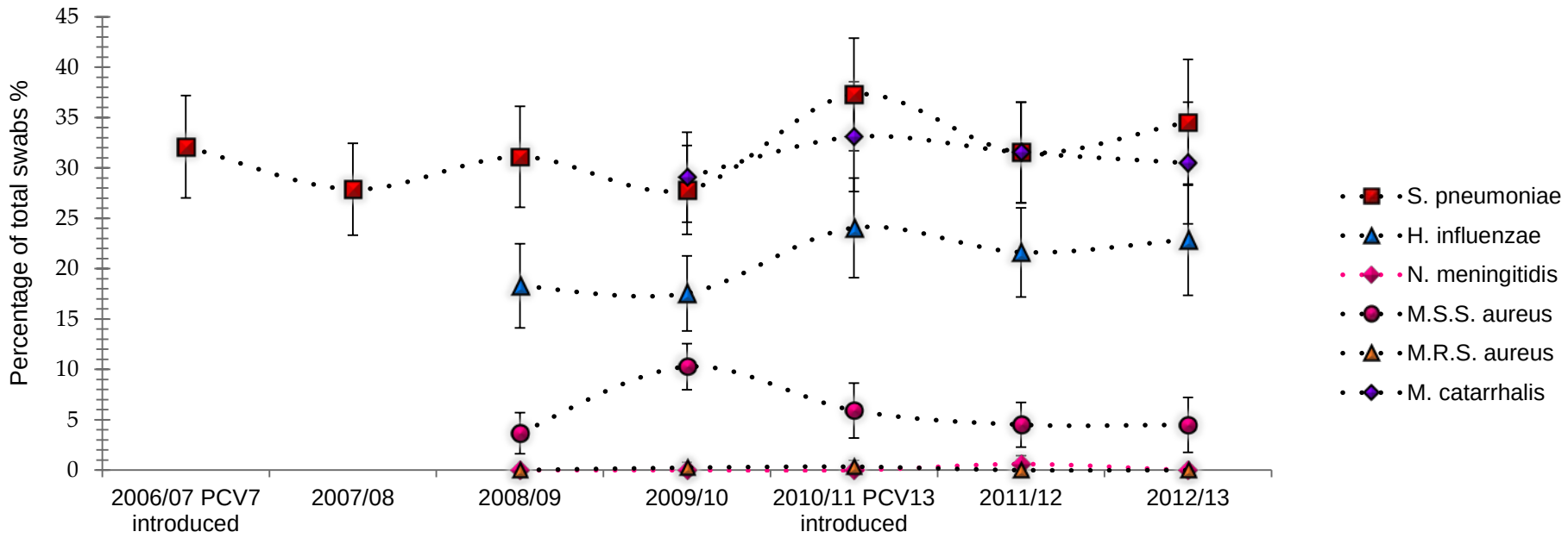


Figure showing the percentage of nasopharyngeal swabs found to be colonised with each of the above bacteria from 2006/07 to 2012/13. Dashed lines are included for visual purposes to link markers for each bacterial species at each time point however data is not continuous and was collected between October to March only per each time-point.

FIGURE 3.2B: PAEDIATRIC NASOPHARYNGEAL SWABS COLONISED WITH SPECIFIC BACTERIAL SPECIES OVER THREE PCV IMPLEMENTATION ERAS

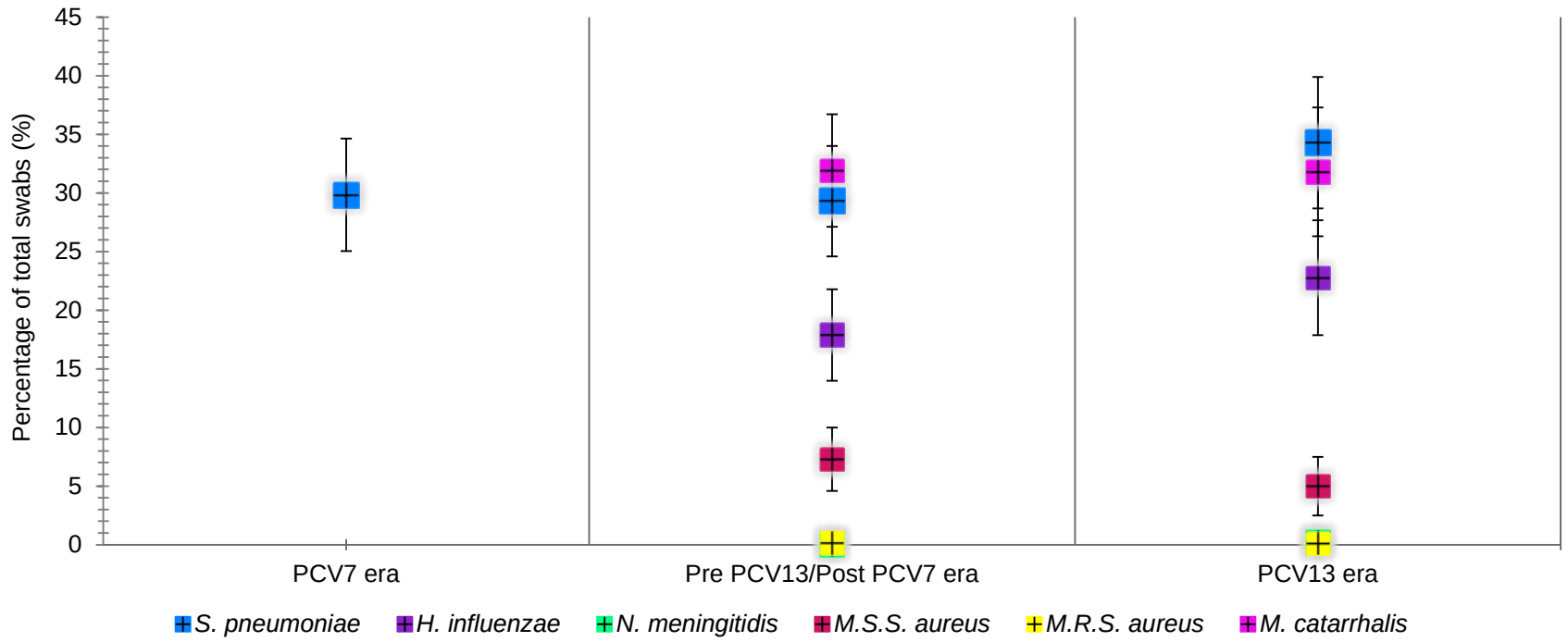


Figure shows the percentage of total paediatric swabs that were colonised with specific bacterial species over three PCV implementation eras. 95% CI error bars are included. PCV7 era was 2006/07 to 2007/08 and included detection for *S. pneumoniae* only. The post PCV7/pre PCV13 era was 2008/09 to 2009/10 and included detection for all bacteria with the exception of *M. catarrhalis* that was first included for detection in 2009/10 only. The PCV13 era was 2010/11 to 2012/13 and included detection of all bacterial species.

3.3.4 CO-COLONISATION

Figure 3.3 demonstrates carriage and co-carriage of *S. pneumoniae* and *H. influenzae*; with or without additional species also being cultured. No significant changes occurred between 2008/09 to 2012/13 for either single species carriage or co-carriage. A non-significant increase in carriage with either *S. pneumoniae* or *H. influenzae* and co-carriage of both species were observed in 2010/11 as compared to the previous and later years of the study. In general, a positive correlation is seen between the two species when carriage and co-carriage rates are recorded.

FIGURE 3.3: PAEDIATRIC NASOPHARYNGEAL SWABS COLONISED WITH *S. PNEUMONIAE* AND *H. INFLUENZAE* TOGETHER AND INDIVIDUALLY

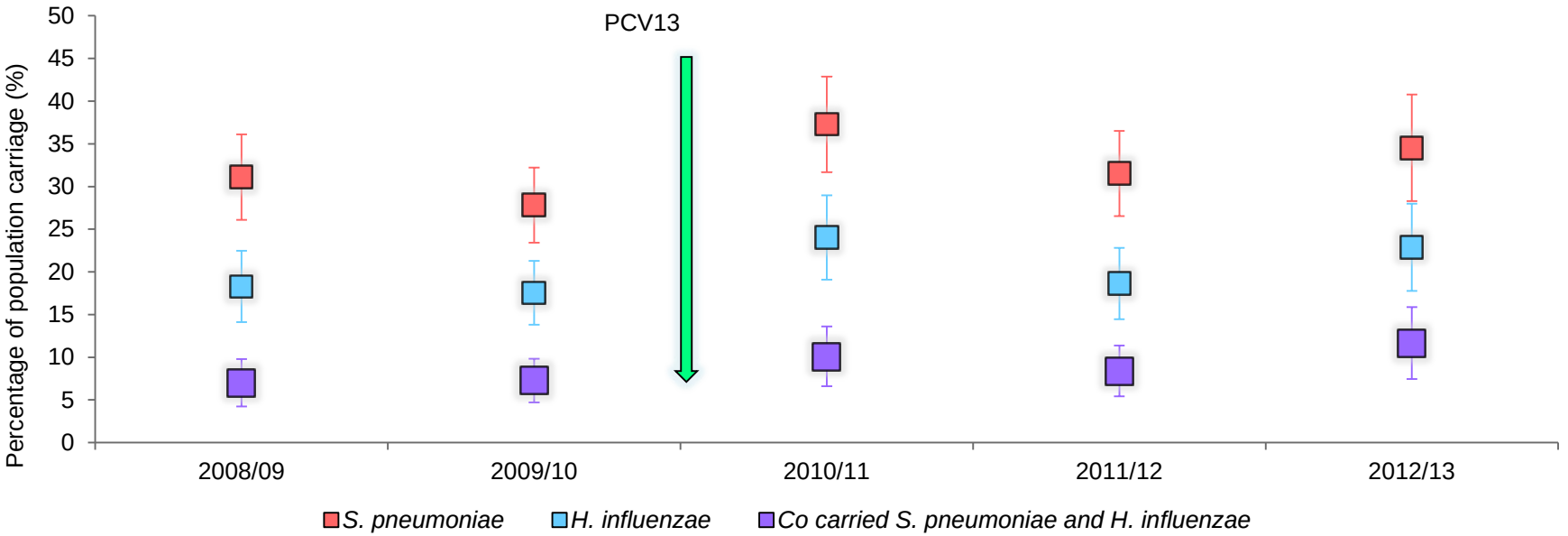


Figure shows the percentage of total paediatric swabs that were colonised with *S. pneumoniae* (red), *H. influenzae* (blue) and instances of co-carriage of both *S. pneumoniae* and *H. influenzae* (purple) from 2008/09 to 2012/13. 95% CI error bars are included. Results shown are a percentage of total population carriage from all swabs taken in each time-point.

3.4 DISCUSSION

In this study population, the mean carriage rate for *S. pneumoniae* was 31.7%, 20.9% for *H. influenzae* and 31.1% for *M. catarrhalis*, in a similar carriage study by Xu et al, the carriage rates were 30.3%, 11.7% and 36.3% respectively, for nasopharyngeal swabs of healthy children (Xu et al., 2012a). Although the two sample populations are very similar, the geography of the children is different and this may have contributed to differences in the percentage values between species.

MSSA showed a significant increase in carriage in 2009/10, and then gradually decreased in carriage from 2010/11 to 2012/13 where the carriage rate was similar to that observed 2008/09. As more studies are aimed towards determining the indirect effects of a PCV vaccine, the body of evidence grows for synergistic and competitive relationships between niche bacteria. However, the relationship between *S. pneumoniae* and *S. aureus* remains unclear, for each study claiming an inverse-relationship existing between *S. aureus* and VT *S. pneumoniae* (Dukers-Muijrers et al., 2013, Bogaert et al., 2004), there is another study claiming no such relationship exists (Lee et al., 2009, Hammitt et al., 2014, Nzenze et al., 2014). These studies focus on carriage changes post-PCV vaccination, either PCV7 or PCV10, in different geographical areas, the exact age of the children in the study also differ. These differences may be crucial in determining a relationship between PCV implementation, the eradication of VT serotypes and the indirect effects on *S. aureus* carriage.

The carriage of the other nasopharyngeal organisms did not change significantly over the study years, regardless of either PCV introduction. However there could be changes at the intra-species level such as changes in serotype prevalence, as seen with *S. pneumoniae* NVT replacement serotypes in carriage as

shown by this group and others (Miller et al., 2011, Tocheva et al., 2011, Pichon et al., 2012, van Gils et al., 2011b, Spijkerman et al., 2012). Serotype-shifting and other forms of genetic diversification for the other species included in the study are not able to be detected at this stage of the study, where organisms are identified to the species level using phenotypic techniques. Due to the study design which selected certain organisms for culture, meaning that, there may be an over-estimate of easy to culture organisms and the growth of fastidious organism may be an under represented. Species of bacteria, which were present in the nasopharynx at lower proportions, may have been missed out by the detection methods, as sensitivity of culture can be less than 100%, particularly when culturing from polymicrobial habitats.

There were no changes in the level of co-carriage between the two species; *S. pneumoniae* and *H. influenzae* and the data does not indicate any significant change since the introduction of PCV13. This observation may currently appear discordant with the hypothesis of this study, which states that an increase in genetic diversification of *H. influenzae* will correlate to an increase in NVT *S. pneumoniae* post-PCV13. The results gained in this chapter do not suggest genetic changes, but suggest that analysis of genetic diversity of *S. pneumoniae* and *H. influenzae* at the genotypic level should be investigated.

Summary:

When considering the bacterial species of interest to this study, no significant changes in the species of bacteria colonizing the nasopharyngeal niche have been observed in the study population since the introduction of PCV13, bearing in mind certain limitations.

The work described in this chapter is based upon standard microbiological culture; carriage data obtained in this way may bias the results towards increased detection of easy to culture organisms as well as bias towards more abundant organisms, while excluding fastidious and low abundance organisms. The time constraints of this study also play an important role, the PCV13 vaccine was only introduced in 2010, and several more years of the study may be required for a population level effect to be observed. Another limitation to this study is that our group does not routinely check for multiple pneumococcal serotypes in a single sample, multiple serotype co-colonisation has not been detected in our samples but it is believed to be under-estimated by our current methodology (Turner et al., 2011).

4. DESCRIPTION OF *S. PNEUMONIAE* CARRIAGE IN THE NASOPHARYNX OF YOUNG CHILDREN DURING THE IMPLEMENTATION OF PCVS IN THE UK

4.1 BACKGROUND

After the introduction of PCV7 several countries reported an increase in NVT IPD (Rodrigo and Lim, 2014, Liesenborghs et al., 2013, Ardanuy et al., 2009). After the implementation of PCV13, the carriage of PCV13 serotypes has reportedly decreased in the UK (van Hoek et al., 2014), whilst reductions in IPD across all age groups have been observed in the US (Moore et al., 2015). However, interest remains in whether or not this is a trend that continues post-PCV13. In a US study post-PCV13, the most common serotypes to cause IPD were 22F (11%), 33F (10%), 38 (9%), 35B (8%), 15B (7%), 19A (7%), 15C (7%), 3 (6%), 23B (5%), and 12F (4%) (Moore et al., 2015). Of these serotypes 22F, 33F, 38, 35B, 15B, 15C, 23B and 12F (together accounting for 56% of IPD) are not included in the PCV13 vaccine. Although use of the PCV13 vaccine has resulted in a reduction in carriage and IPD of VT pneumococci, there is still IPD attributed to PCV13 NVTs. IPD has decreased in the UK from eight cases per 100,000 in the PCV7 era to seven cases per 100,000 post PCV13, this IPD is predominately due to NVTs (Moore et al., 2014). Additionally n the US, it has been described that IPD in children under 5 years of age has decreased a further 64% than just continuing with PCV7 vaccination (Moore et al., 2015). Increases in carriage of certain non-PCV13 VTs have been reported including serogroup 15 in Hong Kong (Ho et al., 2015), and 23B, 15B/C, 16F, 21, 11A, 15A, 6C, 10A, 22F, 35B/F and 23A reported in Greece (Grivea et al., 2014) and 11A, 15A/B/C, 22F, 23A/B and 35B/F reported in Canada (Ricketson et al., 2014). In terms of correlation between IPD causing serotypes and NVTs in carriage in the era following PCV13 vaccination, it can be seen that carriage of serogroup 15 and

serotype 22F, have increased and that these serotypes have been found to cause invasive disease (Levy et al., 2014). For the purpose of this thesis, carried pneumococci (Table 4.1) were characterized to include serotyping, MLST, antibiotic resistance and virulence factor allele detection using a variety of methods. There are many credited and putative virulence factors of *S. pneumoniae* including the *ply* gene, which is ubiquitous to all pneumococci (Jefferies et al., 2010). To test this carriage data-set for emerging serotypes with the potential to cause disease, a list of virulence factors was derived from the genomic sequences of known invasive isolates, and this panel was tested against the carriage data-set using *in silico* methods (see methods section 2.7.5). The purpose of this analysis was to identify carriage strains of pneumococci that form relationships with invasive strains of *S. pneumoniae*, as these strains may have greater ability to cause disease and thus be targets for future PCV formulations. Further work was carried out using mis-match gene results to give a complete virulence factor profile of two strains of prevalent serotype/STs of *S. pneumoniae* to determine patterns of virulence factors within this grouping to avoid under-estimating a strains virulence factor profile.

4.2 OBJECTIVES

- Determine total serotype prevalence of *S. pneumoniae* over a seven year period
- Determine vaccine serotype carriage of *S. pneumoniae* over seven year period
- Determine the effect of *S. pneumoniae* on carriage of other clinically relevant bacterial species
- Determine STs of carried of *S. pneumoniae*
- Determine antibiotic resistance genes and virulence factors of carried *S. pneumoniae* and determine their relatedness to reference pneumococcal genomes

4.3 RESULTS

4.3.1 PNEUMOCOCCAL SEROTYPE DESIGNATION

Several methods were used to determine the serotypes of the pneumococcal isolates during years 2011/12 and 2012/13 of the study (table 4.1). This was done partly as a method comparison and partly due to just one method not being sufficient for all typing needs.

Table 4.1: Method comparison of pneumococcal serotype designation

Analysis method	Results (Number)
Isolates tested	176
Quellung/microarray confirmed	73
microarray only	1
PCR and insilico and serology	45
PCR and insilico only	27
PCR and serology	16
PCR or insilico or serology	7
Conflicting/undetermined	7

Isolate	PCR	Serology	Insilico	Quellung	Microarray	Designated serotype
6007			No Result	**22F**	**22F**	22F
6010			10A	**10A**		10A
6012	10A		10A	**10A**		10A
6015	15A/F	15A +ve	15A/15F			15A
6020			NA	**3**		3
6021	3		3			3
6022			NA	**6C**	**6C**	6C
6023	15A/F	15A +ve	15A/15F			15A
6025			NA	**17F**	**17F**	17F
6026			23A	**23A**		23A
6027			16F	**16F**	**16F**	16F
6032				**15B**	**15B**	15B
6036	23B		23B			23B
6037	6 or 11A/D	11A +ve	11A/11D			11A

6042			23A	**23A**	**23A**	23A
6044	6ABC		6A/B/C/D	**6A**		6A
6052	15B/C	15B +ve	15B/C			15B
6054			15B/15C	**15C**	**15C**	15C
6055	8F/15B	15B +ve	15B/15C			15B
6061	35F/47F	35F +ve	NA			35F
6062	35F /47F	35F +ve	35F/47F			35F
6067	35F/47F	35F +ve	NA			35F
6078	35B	35ABCF+ve/35F +ve	35B			35B
6079	12F/A or 44/46	12 FA -ve	11A/11D	**11A**		11A
6090	8F		NA	**24F**		24F
6094			NA	**not pneumo**	**23B**	23B
6095	35B/38/25F	35B +ve/slight 35F+ve	35B	**35B**		35B
6100	3		NA	**3**		3
6101	19F/12AF/11AD	11A+ve/slight 12F+ve	NA	**11A**		11A
6106	10A or 15B/C	15B+ve	NA			15B
6107	15A/F	15A+ve	NA			15A
6109	15A/F	15A +ve	NA			15A
6110	23B		23B			23B
6112	7F/A		7F/7A	**7F**		7F
6114	6ABC	6AB -ve	NA	**6C**		6C
6117	11A/D or 12F/A	11A +ve	11A/11D			11A
6118	15B/C	15C +ve	NA			15C
6121	10A		10A			10A
6123			No Result	**31**	**31**	31
6126	11A/D or 12F/A	11A+ve	11A/11D			11A
6127	23B	~	23B			23B
6131	15A/F	15A +ve	NA			15A
6133	15A/F	15A weak positive	6C/6D			
6136			31	**31**	**31**	31
6138	15A/F	15A +ve	15A/15F			15A
6142	10A		10A			10A
6148	15B/C	15B +ve	15B/15C			15B
6150	3		6C/6D			
6156	11A/D	11A +ve	11A/11D			11A

6157	15A/F	15A +ve	15A/15F			15A
6160	3		3			3
6171	23A		23A			23A
6175			35F/47F	**35F**	**35F**	35F
6186			NA	**15A**		15A
6188	3		3			3
6189	11A/D	11A +ve	NA			11A
6191	5		35F/47F			
6192	23B		23B			23B
6197	23F or 15B/C	15C +ve	15B/15C			15C
6200	33FA/37/ 34/35AC/ 42	33F +ve	No Result			33F
6206	6ABC		6C/6D	**6C**		6C
6207	11A/D	Left out of sera list	NA	**11A**		11A
6208	23A		23A			23A
6214	16F/19A/ 22FA	22F +ve	No Result			22F
6216	23B		23B			23B
6221	10A or 42 or 35A/C	35A/C -ve	10A	**10A**		10A
6224			34	**34**	**DNA too low**	34
6228	6ABC		6C/6D	**6C**		6C
6230	8 or 23B		34	**34**		34
6231	23B		23B			23B
6232	5		35F			
6233	11A/D or 25F or 38	11A/D -ve	38/25F/25 A	**38**		38
6237	19A		19A			19A
6238	21		21			21
6241	15B/C	15C +ve	15B/15C			15C
6247	7A/f or 23A		23A	**23A**		23A
6248	15A/F	15A +ve	15A/15F			15A
6249	6ABC	6AB -ve	6C/6D	**6C**		6C
6251	10A		10A			10A
6253	19F or 12ABF		NA	**11A**		11A
6255			No Result	**JH**	**DNA too low**	
6260	38 or 25F		35B	**35B**		35B
6272	23B		23B			23B

6274	3 or 19A		11A/11D	**11A**		11A
6276	38 or 25F		35B	**35B**		35B
6287	6		6C/6D	**6C**		6C
6288	12F/A or 44/46	12 FA -ve	11A/11D	**11A**		11A
6302	21		NA			
6304	~		31	**31**	**31**	31
6311	23B		23B			23B
6312	23B		23B			23B
6313	21		NA			
6318	15B/C	15C+ve	NA			15C
6320	7F/A or 23A/F		NA	**23A**		23A
6326	35B	35B +ve	35B			35B
6328	21		NA			
6329	23B		23B			23B
6331	11A/D or 23B	11A +ve	11A/11D			11A
6332	35A/F or 37	35A-F -ve	15A/15F	**15A**		15A
7001	35B	35B +ve	35B			35B
7004	15A/F	15A-F -ve	35F/47F	**35F**		35F
7005	22F/16F	22F -ve	16F	**16F**		16F
7006	22F/16F	22F -ve	16F	**16F**		16F
7010	33F/15AF	15A +ve	15A/15F			15A
7014	22F/16F	22F-ve	No result	**22F**		22F
7015	19F		21			
7016	23F/15B	15B -ve	15B/15C	**15B**		15B
7023	15A/F	15A+ve	15A/15F			15A
7026	21		21			21
7027	15A/F	15A +ve	15A/15F			15A
7028	35B/23B	15B-ve	35B	**35B**		35B
7033	23F		No result	**NT**		NT
7041	15A/23B	15A +ve	15A/15F			15A
7050	23A / 23B		23A	**23A**		23A
7051	38/25F/35B	35ABC -ve	38/25F/25A	**38**		38
7052	23B		NA			
7053	23B		23B			23B
7059	23F/15B	15B -ve	15B/15C	**15C**		15C
7060	11A/D	11A +ve	11A/11D			11A
7062	16F/22F	22F -ve	16F	**16F**		16F
7064	6ABC	6AB -ve	6C/6D	**6C**		6C
7065	23F/15B	15b -ve	15B/15C	**15B**		15B
7067	6AB	6A +ve	6A/B/C/D			6A

7068	6AB	6A +ve	NA			6A
7069	15A	15A +ve	15A/15F			15A
7070	15A	15A +ve	15A/15F			15A
7073	6ABC	6AB -ve	NA	**6C**		6C
7075	7F/23A		23A	**23A**		23A
7076	35B/25F/38	35ABC-ve, 35F +ve	35B	**35B**		35B
7079	12F/11A/D	11A+ve	11A/11D			11A
7084	23B		23B			23B
7085	11A/D or 12F/A or 38	12F +ve	No result			12F
7091	12F/A	12FA -ve	11A/11D	**11A**		11A
7093	6ABC/33F	6AB -ve/ 33F -ve	33F/33A/37	**33F**		33F
7102	6ABC	6 -ve	6C/6D	**6C**		6C
7103	23B		NA			
7104	35B	35B +ve	35B			35B
7105	21		NA			
7106	24ABF	24F +ve	24A/24B/24F			24F
7107	15A/33F	15A +ve	15A/15F			15A
7111	15BC	15BC -ve	10A	**10A**		10A
7116	23F/15B/10A	15B +ve	15B/15C			15B
7118	31??		31	**31**		31
7119	12FA/44/46/19F	12FA-ve	10A	**10A**		10A
7123	23F/15B/10A	15B +ve	No result			15B
7126	12F/A	12FA -ve	11A/11D	**11A**		11A
7136	35ABC	35B +ve	35B			35B
7139	15A	15A +ve	15A/15F			15A
7140	34/9N/35AC	35 -ve	34	**34**		34
7141	21		21			21
7143	23F/15B	15B-ve	15B/15C	**15B**		15B
7144	24ABF	24F +ve	24A/24B/24F			24F
7146	23F/15B	15B-ve	15B/C	**15C**		15C
7151	35ABC	35B +ve	35B			35B
7159	35ABC	35B +ve	35B			35B
7161	23B		23B			23B
7166	24ABF	24B +ve	35F/47F			
7172	12F/A	12FA -ve	NA	**11A**		11A

7175	24ABF	24F +ve	24A/24B/24F			24F
7176	35F/17F	35F +ve	35F/47F			35F
7179	15B/C	15B +ve	15B/15C			15B
7181	15B/C	15BC -ve	10A	**10A**		10A
7184	33F/15A	15A +ve	15A/15F			15A
7186	24F	24F +ve	24A/24B/24F			24F
7188	33F	33F -ve	33F/33A/37	**33F**		33F
7189	33F	33F -ve	33F/33A/37	**33F**		33F
7192	6ABC	6AB -ve	6C/6D	**6C**		6C
7193	23B		23B			23B
7194	23B		23B			23B
7206	10A		No result			
7207	15B/C	15B +ve	No result			15B
7217	15A/33A	15A +ve	15A/15F			15A
7218	22F/19A/16F	22F +ve	No result			22F
7220	15B/C	15B +ve	15B/C			15B
7221	15B/C or 10A	15B +ve	15B/C			15B
7223	23F		NA	**15B**		15B

4.3.2 TOTAL PNEUMOCOCCAL CARRIAGE IN UK CHILDREN LESS THAN FIVE YEARS OF AGE

Pneumococcal carriage has been recorded from the study cohort of the paediatric outpatients department of the Southampton General Hospital since 2006/07 to 2012/13, carriage and pneumococcal serotype per each year has been grouped into Table 4.2 in order of PCV inclusion and abundance detected over the seven study time-points. The study began shortly after PCV7 introduction to the UK in 2006/07, carriage was recorded as being dominated by PCV7 VTs with 6B, 19F and 23F accounting for 24, 13 and 9 isolates (7.41%, 4.01% and

2.78% of total carriage), the pre-PCV13 VT 6A accounted for 10 isolates (3.09% of total carriage) (figure 4.1.a). In the 2007/08, all carriage of all PCV7 VTs decreased, 6B and 19F carriage decreased to 15 and 6 isolates (4.02% and 1.61%) respectively. Carriage of 6A increased 0.93% to 15 isolates (4.02%), but an increase of 2.82% from 3 to 14 isolates (3.75%) was detected for PCV13 NVT 6C (figure 4.1.b). During the study period 2008/09 further decreases in carriage were seen for PCV7 VTs, with increases in carriage for all PCV13 VTs bar 6A where a 2.17% decrease in carriage from 15 to 6 isolates was detected. In terms of PCV NVTs, increases in carriage were detected for 6C, 23B, 22F, 23A and NT *S. pneumoniae* (figure 4.1.c). The study period 2009/10 was the last period prior to PCV13 introduction and no carriage of any PCV7 VTs was detected with the exception of a small proportion of serotypes 6B and 23F, 2 and 3 isolates each (1.25% total). PCV13 VTs 1, 3, 6A, 7F and 19A remained in carriage with 1, 2, 5, 2 and 12 isolates respectively (5.5% total). PCV13 NVT 6C decreased from 14 to 4 isolates (3.27%), but serotypes 11A, 21 and 35F increased to 13, 10 and 7 isolates (1.13%, 1.6% and 1.39% increases) respectively (figure 4.1.d). By 2010/11, carriage of PCV7 VTs apart from 6B (1 isolate) was eradicated, and carriage of all PCV13 VTs was reduced. Carriage of PCV13 NVTs 6C, 21 and 35F increased to 9, 13 and 9 isolates (3.14%, 4.53% and 3.14% of total carriage), respectively (figure 4.1.e). In 2011/12 all PCV7 VT carriage was eradicated, PCV13 VT carriage was also reduced bar a spike in carriage of serotype 3, with an increase to 5 isolates (1.15% increase). PCV13 NVTs 11A, 23B and 15A, were at 12, 11 and 10 isolates ease (represented 3.6%, 3.3% and 3% of total carriage) (figure 4.1.f). During the 2012/13 study-period, PCV VTs were not detected and all PCV13 VT carriage was reduced bar 2 incidences (0.9%) of carriage of serotype 6A. PCV13 NVT carriage of 15B, 23B, 15A and 35B dominated total carriage with detection reaching 10, 7, 10 and 7 incidences (4.48%, 3.14%, 4.48% and 3.14%, respectively) (figure 4.1.g). In figure 4.1.g, total carriage of

pneumococcal serotypes is shown with PCV13 NVTs 6C and 11A being detected in more abundance over the seven year study time-points that PCV7/PCV13 VTs.

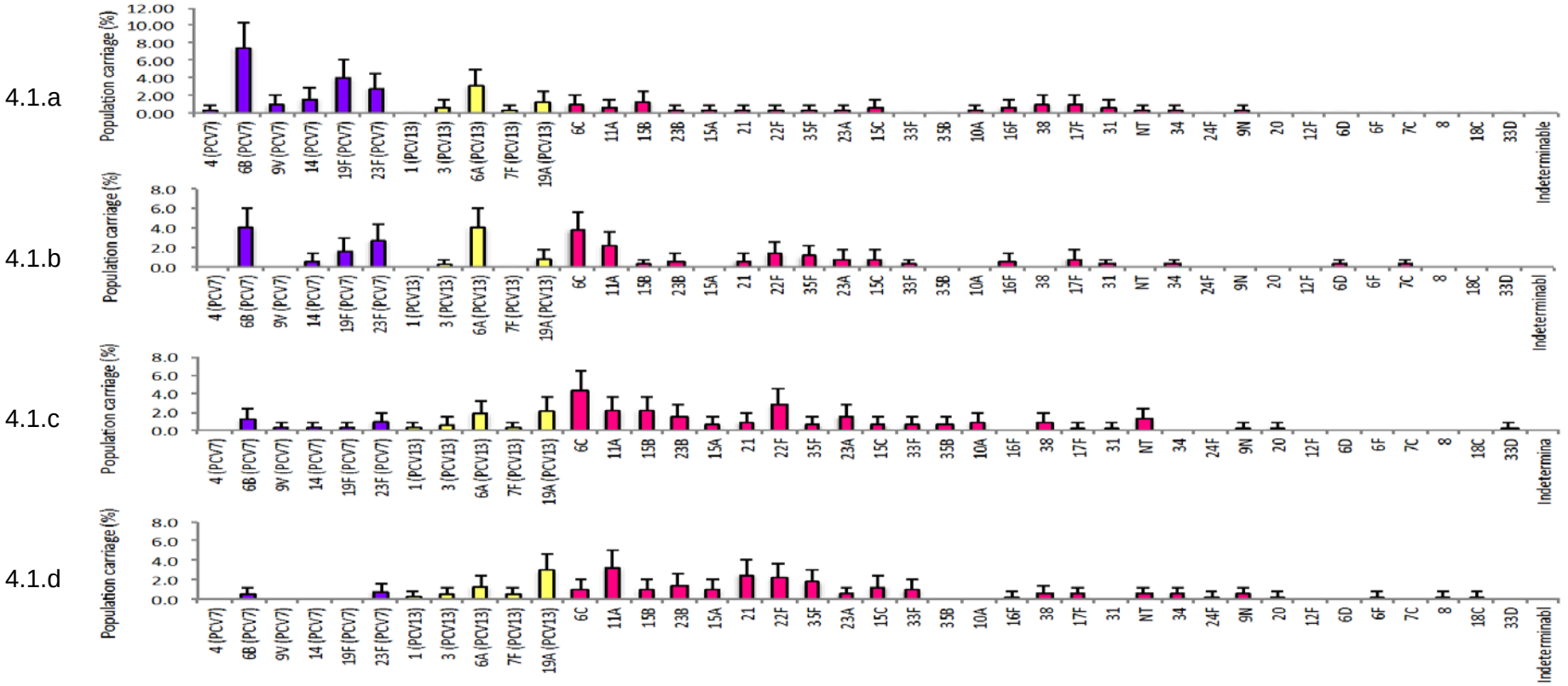
Table 4.2: Pneumococcal serotypes detected in each study year

Serotype	06/07	07/08	08/09	09/10	10/11	11/12	12/13	Total
4 (PCV7)	1	0	0	0	0	0	0	1
6B	24	15	4	2	1	0	0	46
9V	3	0	1	0	0	0	0	4
14	5	2	1	0	0	0	0	8
19F	13	6	1	0	0	0	0	20
23F	9	10	3	3	0	0	0	25
1	0	0	1	1	1	0	0	3
3	2	1	2	2	1	5	0	13
6A	10	15	6	5	2	1	2	41
7F	1	0	1	2	1	1	0	6
19A	4	3	7	12	5	1	0	32
6C	3	14	14	4	9	6	4	54
11A	2	8	7	13	7	12	5	54
15B	4	1	7	4	7	5	10	38
23B	1	2	5	6	8	11	7	40
15A	1	0	2	4	6	10	10	33
21	1	2	3	10	13	4	3	36

22F	1	5	9	9	3	2	2	31
35F	1	4	2	7	9	4	2	29
23A	1	3	5	2	4	6	2	23
15C	2	3	2	5	2	5	2	21
33F	0	1	2	4	8	1	3	19
35B	0	0	2	0	3	5	7	17
10A	1	0	3	0	1	6	4	15
16F	2	2	0	1	4	1	3	13
38	3	0	3	2	1	1	1	11
17F	3	3	1	2	0	1	0	10
31	2	1	1	0	2	3	1	10
NT	1	0	4	2	0	0	1	8
34	1	1	0	2	0	2	1	7
24F	0	0	0	1	0	1	4	6
9N	1	0	1	2	0	0	0	4
20	0	0	1	1	1	0	0	3
12F	0	0	0	0	1	0	1	2
6D	0	1	0	0	0	0	0	1
6F	0	0	0	1	0	0	0	1
7C	0	1	0	0	0	0	0	1
8	0	0	0	1	0	0	0	1
18C	0	0	0	1	0	0	0	1
33D	0	0	1	0	0	0	0	1
Untyped	0	0	0	0	0	5	2	7

Pneumo total	103	104	102	111	100	99	77	696
Carriage totals	324	373	328	399	287	333	223	2267

FIGURE 4.1 A-D TOTAL SEROTYPE PREVALENCE IN HAMPSHIRE CHILDREN LESS THAN FIVE YEARS OF AGE, A: 2006/07, B: 2007/08, C: 2008/09, D: 2009/10



Pneumococcal serotype carriage per year, shown as a percentage of all bacterial carriage from each study time-point, data is presented by PCV7 inclusion (blue), PCV13 inclusion (yellow) and by abundance of PCV13 NVTs (pink) detected over the seven study time-points. Error bars – 95% CI.

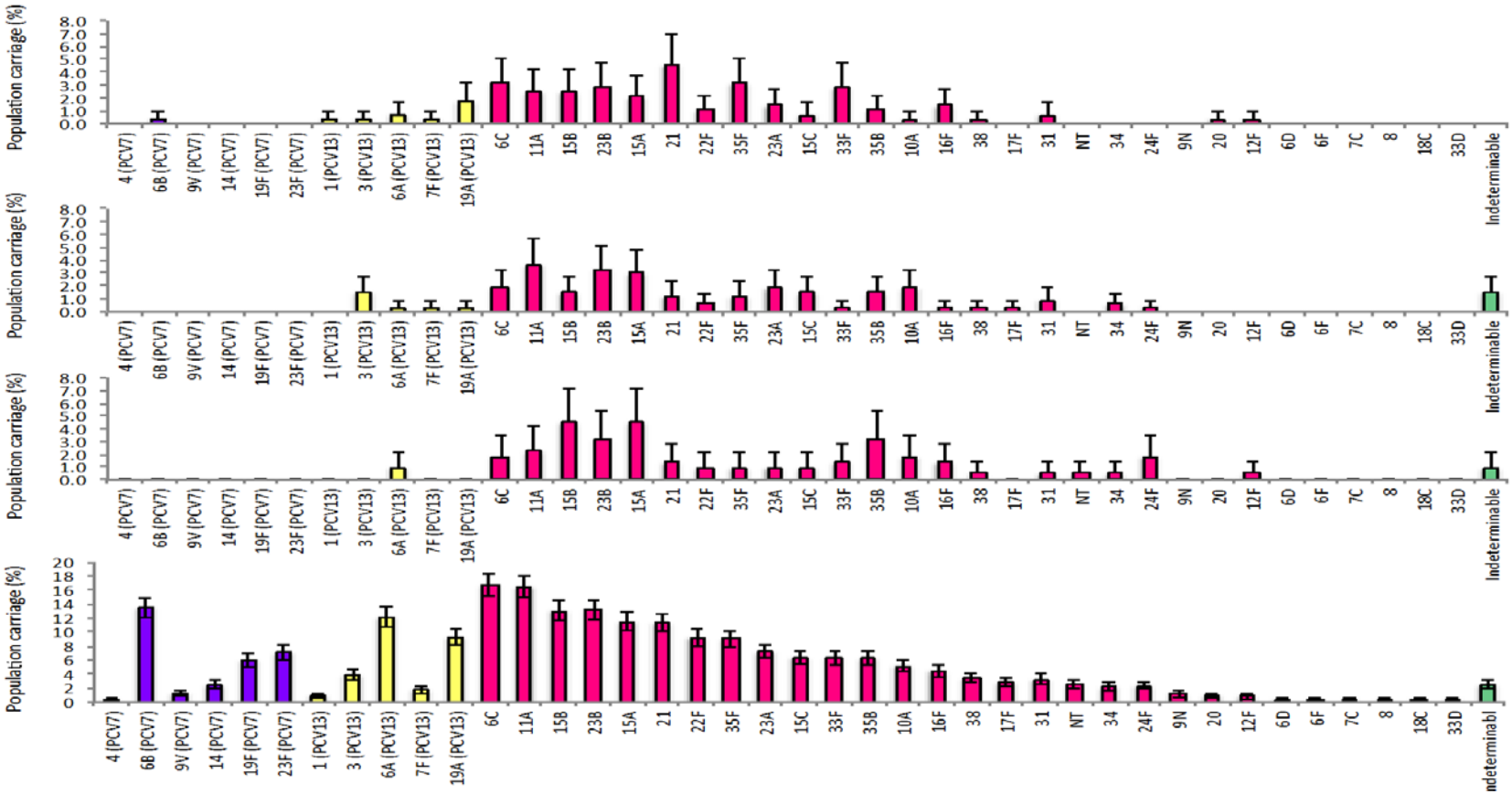
FIGURE 4.1 D-G TOTAL SEROTYPE PREVALENCE IN HAMPSHIRE CHILDREN LESS THAN FIVE YEARS OF AGE, D: 2010/11, E: 2011/12, F: 2012/13, G: 2006-2013

4.1.d

4.1.e

4.1.f

4.1.g

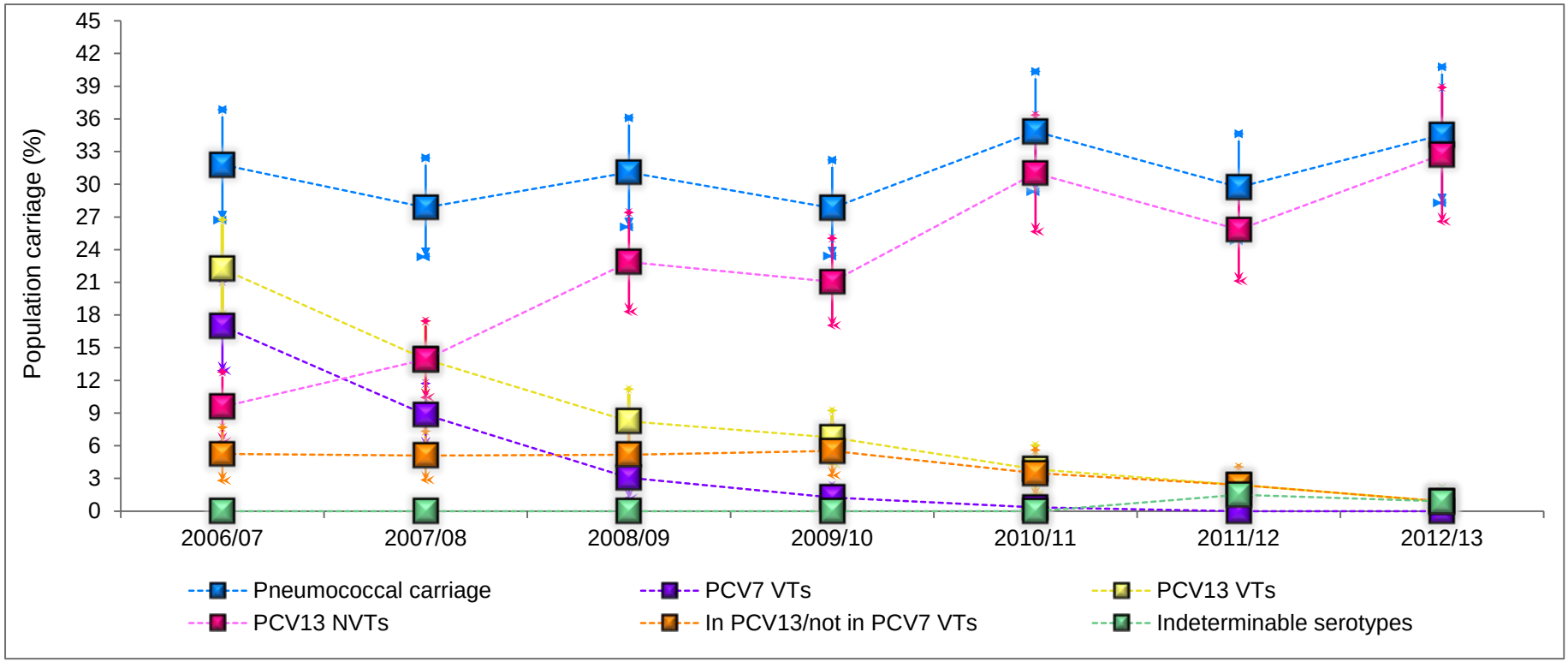


Pneumococcal serotype carriage per year, shown as a percentage of all bacterial carriage from each study time-point, data is presented by PCV7 inclusion (blue), PCV13 inclusion (yellow) and by abundance of PCV13 NVTs (pink) detected over the seven study time-points. Error bars – 95% CI. Reduction in VT carriage over duration of study shown with VT replacement in later study years.

4.3.2 VACCINE TYPE SEROTYPE CARRIAGE OF *S. PNEUMONIAE* OVER SEVEN YEAR PERIOD

When specific pneumococcal serotypes aren't taken into consideration, pneumococcal carriage remains stable over a seven-year period (figure 4.2). However at the serotype level, many changes occur. PCV7 VTs decrease steadily from 2006/07, where they made up almost 17% of the 32% pneumococcal carriage proportion, to 0% in both 2011/12 and 2012/13. PCV13 VTs not included in PCV7 contribute to an additional 5% of carriage and only begin to decrease post-PCV13 vaccine implementation. PCV13 VTs to include the PCV7 VTs and 6 additional PCV13 serotypes were seen to steadily decrease over the study periods, by 2012/13 they almost disappeared from carriage. While these decreases in VT carriage have occurred, the overall carriage rate has remained steady due to increases in PCV NVT serotypes. In 2006/07, NVTs contributed to 9.5% of carriage and climbed steadily to 32.7% carriage in 2012/13. The total pneumococcal carriage rate was 34.5% in 2012/13; just 2.06% of pneumococcal carriage was caused by a serotype not assigned as a PCV NVT.

FIGURE 4.2 POPULATION CARRIAGE OF PCV VACCINE TYPE SEROTYPES EACH STUDY YEAR (2006/07 TO 2012/13)



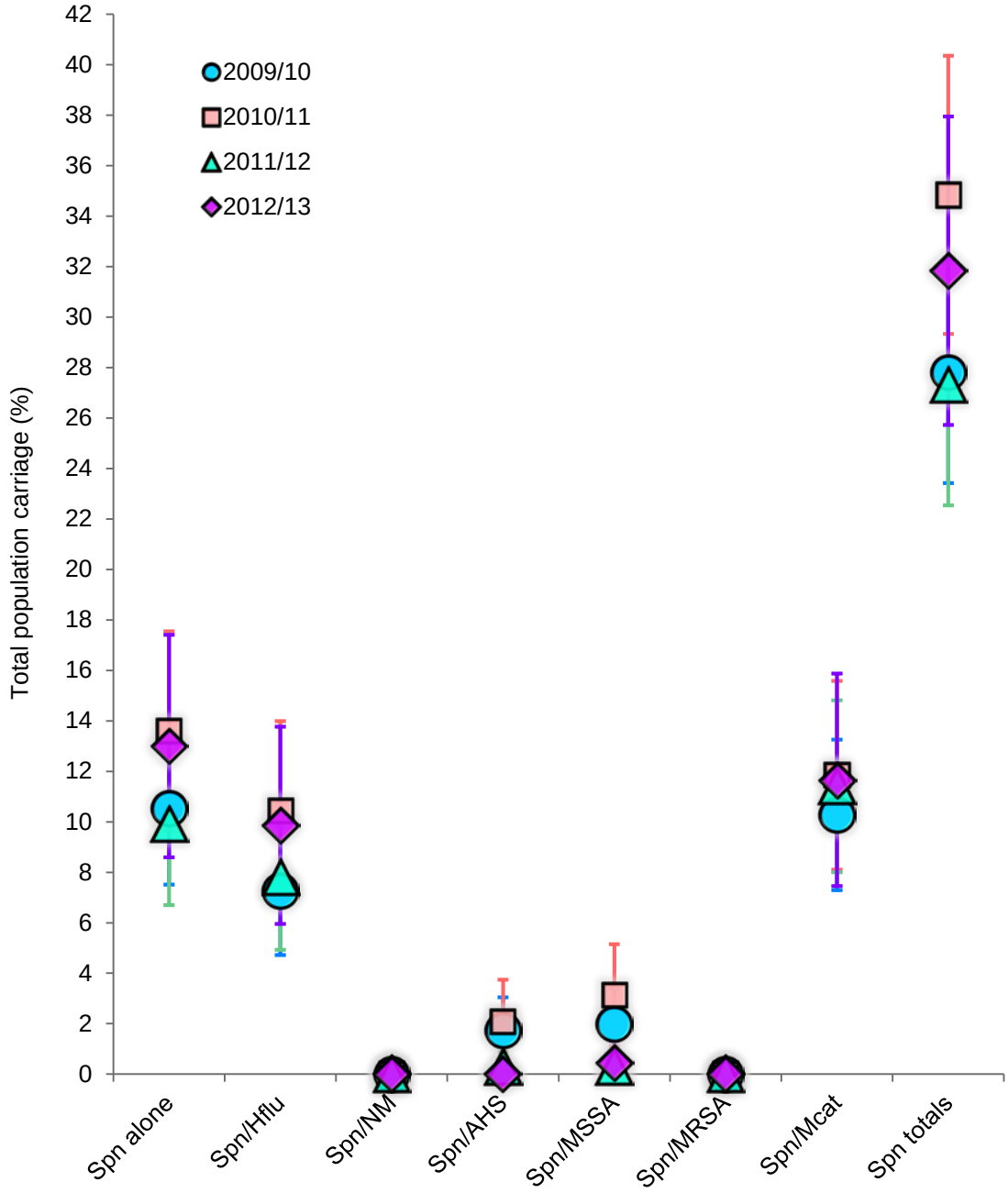
Carriage of pneumococcal isolates presented as a percentage of the total population's bacterial carriage, strains were typed and segregated into groups dependent upon their inclusion/exclusion of PCV formulations. Pneumococcal carriage includes all isolates that were initially detected by culture (blue), isolates typed as PCV7 serotypes (purple), isolates typed as PCV13 VTs that weren't included in PCV7 (orange), PCV13 VTs, which are a total of PCV7 VTs and PCV13 VTs that weren't included in PCV7 (yellow), all isolates typed as PCV13 NVTs (pink). Isolates that could not be defined as a single confirmed serotype are also included as they contribute to overall pneumococcal carriage (green). Error bars – 95% CI.

4.3.3 THE EFFECT OF PCV13 VACCINE SEROTYPES OF *S. PNEUMONIAE* ON CARRIAGE OF OTHER CLINICALLY RELEVANT BACTERIAL SPECIES

Four study time-points from 2009/10 to 2012/13 also included detection of *H. influenzae*, *N. meningitidis*, non-pneumococcal alpha haemolytic streptococci, *S. aureus* and *M. catarrhalis*. The dataset was analysed for co-carriage of *S. pneumoniae* with these other species of bacteria. The study results indicate that *S. pneumoniae* was found to be carried alone ranging between 9.91% and 13.59% of the total bacterial carriage percentage over all study years (2006/07 – 2012/13), study-periods 2009/10 and 2011/12 had the least pneumococcal carriage on its own, with study-periods 2010/11 and 2012/13 having the most pneumococcal-only carriage. A similar trend was seen with *S. pneumoniae* being co-carried with *H. influenzae* more frequently during 2010/11 and 2012/13 with co-carriage being detected ranging between 7.81% and 10.45% of the total carriage percentage in study years 2008/09 to 2012/13. *S. pneumoniae* and *M. catarrhalis* were co-carried between 10.8% and 11.66% throughout the four study time-points (2009/10 – 2012/13); variation between different study periods was not detected. *S. pneumoniae* was co-carried with other AHS between 0% and 2.09% of total carriage but only in study time-points 2009/10, 2010/11 and 2012/13. Similar results were observed for co-carriage of *S. pneumoniae* and MSSA with 0% to 3.14% of total carriage percentage, co-carriage was only detected in study periods 2009/10 and 2010/11. In the 2011/12 study period, *S. pneumoniae* was carried either alone, or with *H. influenzae* or *M. catarrhalis*, and during the 2012/13 time-point but with the addition of carriage with other alpha haemolytic streptococci, Co-carriage with meticillin resistant *S. aureus* or *N. meningitidis* was not detected as neither of these two species were detected in carriage during any of the four study time-points. Total pneumococcal carriage ranged between 27.33% to 34.84% of the total population

carriage, with the highest carriage detected in study year 2010/11, co-carriage was also highest during this study year. Pneumococcal carriage in study period 2012/13, was the second highest percentage but lower co-carriage was detected for alpha haemolytic streptococci and MSSA.

FIGURE 4.3 CO-CARRIAGE BETWEEN *S. PNEUMONIAE* AND *H. INFLUENZAE*, N. MENINGITIDIS, NON-PNEUMOCOCCAL ALPHA HEAMOLYTIC STREPTOCOCCI, *S. AUREUS* AND *M. CATARRHALIS* (2009/10 TO 2012/13)

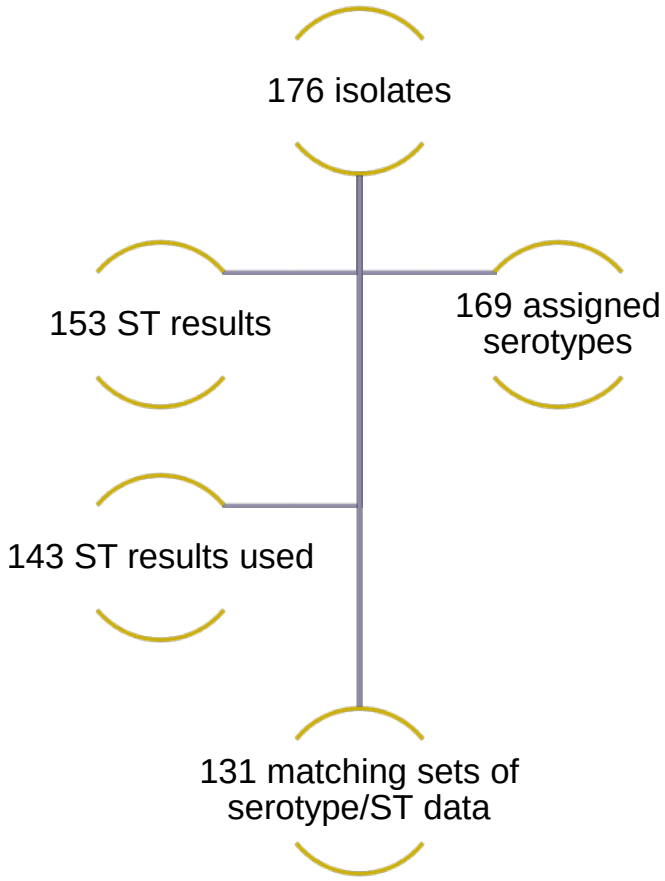


Carriage of *S. pneumoniae* on its own (Spn alone), with *H. influenzae* (Spn/Hflu), with *N. meningitidis* (Spn/NM), with other non-pneumococcal alpha haemolytic streptococci (Spn/AHS), with MSSA (Spn/MSSA) and MRSA (Spn/MRSA) *S. aureus* and with *M. catarrhalis* (Spn/Mcat). Total percentages of *S. pneumoniae* carriage were also recorded (Spn totals). Results are presented as a percentage of total population carriage. Error bars: 95% CI.

4.3.4 CARRIAGE OF PNEUMOCOCCAL GENOTYPES AS DETERMINED BY MLST

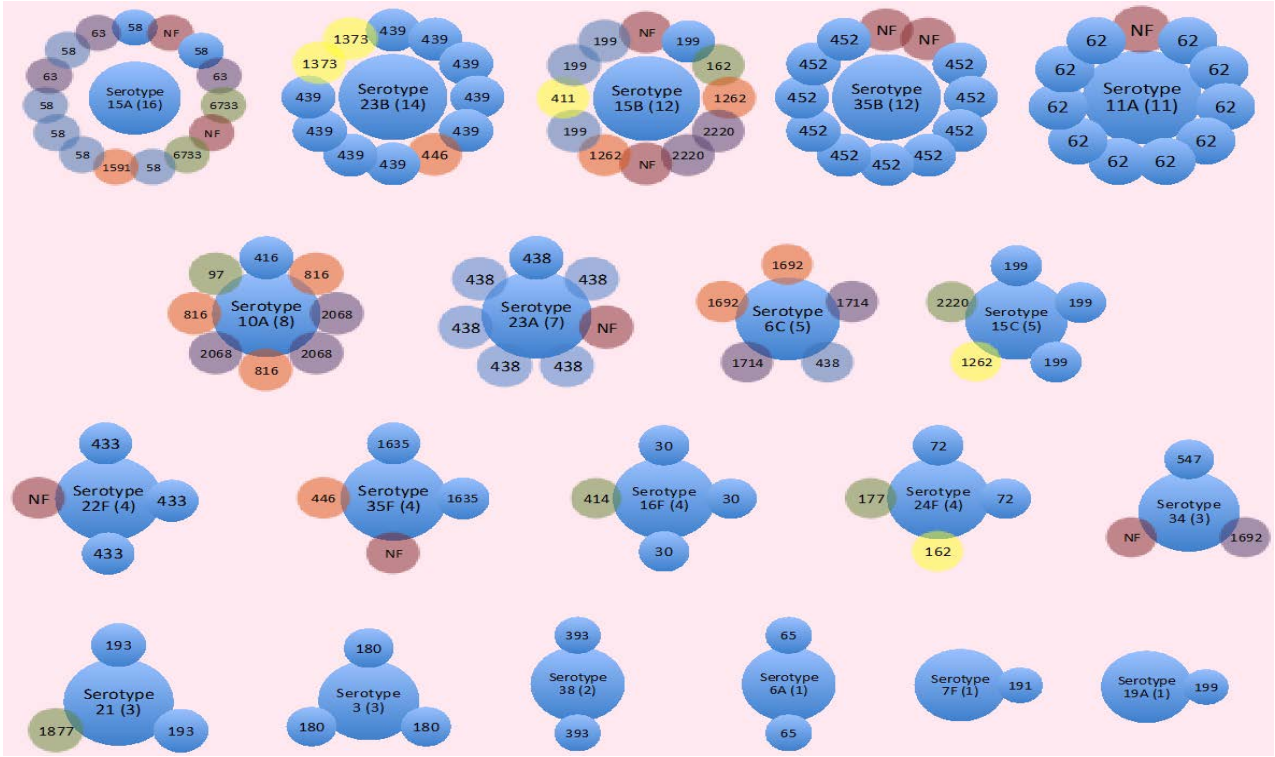
S. pneumoniae isolates from study years 2011/12 and 2012/13 were typed using a range of methods to produce serotype and sequence type (ST) results. Isolates that underwent both typing methods were used to determine patterns between serotype and associated genotypes. In the data-set (figure 4.4), 20 serotypes were characterised, producing 38 assigned STs and 10 novel STs (NF). In the two study years, 15A, 23B, 15B, 35B and 11A were the most prevalent serotypes detected. STs 439, 452 and 62 were the most prevalent STs detected and were associated with serotypes 23B, 35B and 11A respectively (figure 4.5). Of the 20 serotypes, 5 serotypes (3, 38, 6A, 7F, 19A) each had one distinct ST (180, 393, 65, 191, 199), 6 serotypes (35B, 11A, 23A, 22F, 16F, 21) each had 2 distinct STs (452 and NF, 62 and NF, 438 and NF, 433 and NF, 30 and 414, 193 and 1877), 6 serotypes (23B, 6C, 15C, 35F, 24F, 34) each had 3 distinct STs (439, 1373 and 446/1692, 1714 and 438/199, 220 and 1262/ 1635, 446 and NF/72, 177 and 162/547, 1692 and NF), 1 serotype (10A) had 4 distinct STs (2068, 816, 97 and 416), 1 serotypes (15A) had 5 distinct STs (58, 63, 6733, 1591 and NF) and 1 serotype (15B) had 6 distinct STs (199, 162, 1262, 2220, 411 and NF). When analysing genetic relatedness of pneumococcal STs (figure 4.6) there were 138 STs assigned and 41 of these STs were distinct, groups were assigned if 6 out of the 7 loci were identical, 6 sub-groups were formed based on this criteria and a 7th group comprising of distinct singletons was also formed. Two STs were assigned as predicted founders for subsequent STs, ST816 was classed as the predicted founder of both ST2068 and ST461, ST411 was classed as the predicted founder of both ST199 and ST2220.

FIGURE 4.4 SEROTYPING AND MLST RESULTS OF PNEUMOCOCCAL ISOLATES (2011/12 – 2012/13)



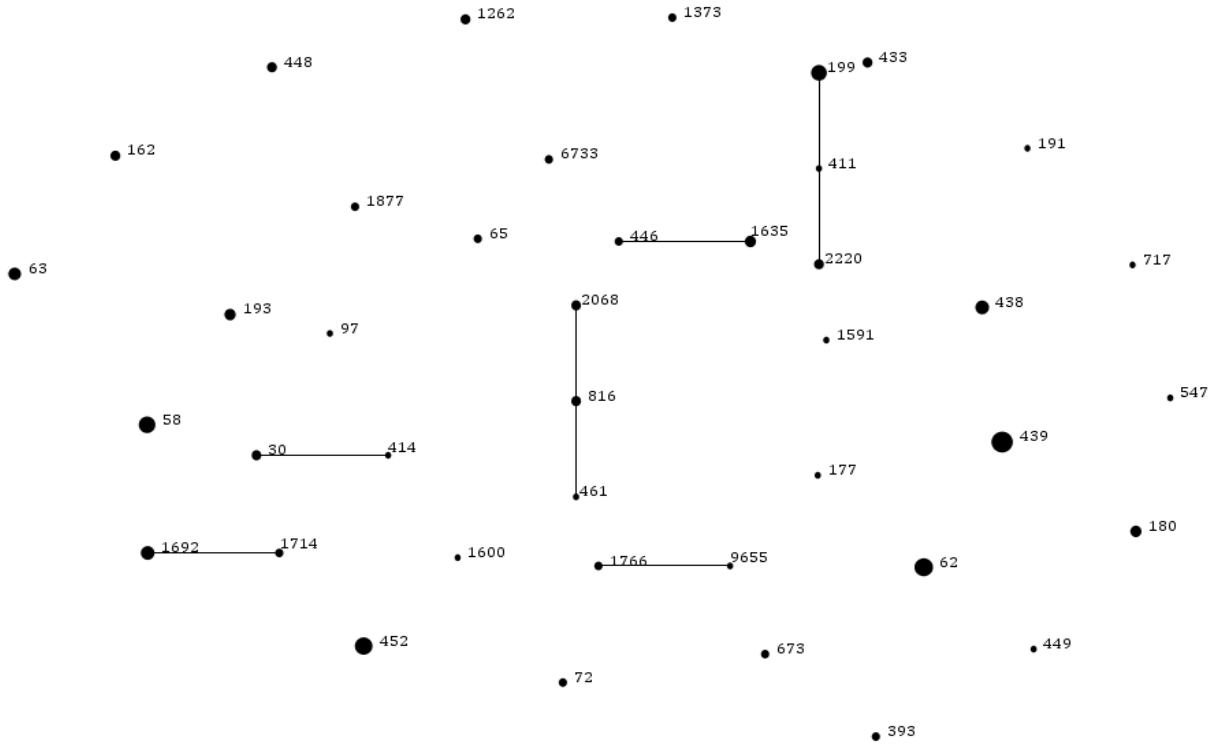
Of the 176 isolates, 7 could not be assigned to a definitive serotype so were excluded from this analysis. 7 isolates proved difficult to culture so were removed from this analysis. 153 ST results were received. 10 were excluded due to sequencing quality. 13 novel allelic combinations were encountered and are included as ST NF (not found). Of the 131 matching sets of serotype and ST data there were 20 distinct serotypes comprising of 38 distinct STs and 10 novel STs termed NF.

FIGURE 4.5 RELATIONSHIP BETWEEN CARRIED PNEUMOCOCCAL SEROTYPES AND ASSOCIATED STS (2011/12 – 2012/13)



Each serotype is shown as a blue circle with the number of isolates attributed to that serotype in the center, surrounding these blue circles are smaller circles with the associated ST results. Multiple STs of the same value are included as single circles of the same colour; additional STs are labeled with another colour. STs that could not be assigned were labeled NF and are denoted by a small red circle. Serotypes are listed in descending order of prevalence.

FIGURE 4.6 EBURST ANALYSIS OF 138 STS OF *S. PNEUMONIAE* (2011/12 – 2012/13) BASED ON SEVEN HOUSEKEEPING GENES USED IN MLST ANALYSIS



Of the 138 ST results, 41 distinct STs are marked above by black circles; circle size denotes the number of isolates that were assigned that ST. Black lines between STs indicate that there are six shared alleles between different STs and hence indicates genetic relatedness. Where there are 3 STs joined by a line, the ST in the centre is the predicted founder of that sub-group. Data shows carried isolates are genetically distinct.

4.3.5 ANTIBIOTIC RESISTANCE GENES AND VIRULENCE FACTORS OF CARRIED *S. PNEUMONIAE* AND THEIR RELATEDNESS TO VIRULENT PNEUMOCOCCAL STRAINS

Using online resources of ResFinder, a tool for detecting antibiotic resistance genes, (https://cge.cbs.dtu.dk/services/ResFinder/), and the Virulence Factor Data Base (VFDB), a tool for detecting virulence factors in sequenced strains of bacteria, (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Streptococcus), the data-set was analysed to detect potential genes of antibiotic resistance. Briefly, gene fragments virulence genes (table 4.3) and of antibiotic resistance genes (table 4.4) were compiled into a data-file and were used along with short read sequence typing (SRST2) to detect the presence of these exact gene fragments taken from reference genomes. This method allows a user to compile their own genes/markers of interest relevant to the data set but this method also relies on sequence data of these genes already being available to use from a database such as Genbank. The genomic sequences from study periods 2011/12 and 2012/13 were examined for matching gene fragments to the antibiotic resistance genes and alleles from different virulence factors derived from the genomes of Table 4.5. For the purpose of this results chapter, exact matches only were used for the entire data-set. The option of mis-matches is available, however due to the volume of data gained when using this method, it was decided that the analysis of a small number of strains only would be investigated, with the focus on determining the virulence factors of certain STs of interest present in both study years (2011/12 and 2012/13). The purpose of this work is to determine the presence of selected antibiotic resistance determinates '*in silico*'. Information resulting from this analysis would provide the basis for confirmatory phenotypic analysis. Penicillin resistance was not able to be determined through searching for a set panel of genes so was not included in this study, penicillin resistance is mediated through an array of genes, some of which are penicillin

binding proteins (*pbp*) but some are not (Fani et al., 2011), mutations frequently occur within these genes also (Aslan et al., 2012). Use of *pbp* genes using the method of SRST2 proved unsuccessful in detecting resistance within these isolates – no positive results were returned.

Table 4.3 Gene names and details of pneumococcal virulence factors found in data set, gene details in Appendix 2.1

Gene	Details
eno	enolase surface binding plasminogen
ply	pneumolysin
psaA	manganese transporter lipoprotein
piuA	iron transporter lipoprotein
htrA/degP	heat-shock induced serine protease
lytA	autolysin gene
srtB	pillus associated sortase
srtC	pillus associated sortase
cbpG	choline binding protein
lytC	cell wall lysozyme protein
lmb	adhesion lipoprotein
tig/ropA	protease trigger factor
nanA	surface adhered neuraminidase protein
slrA	rotamase lipoprotein A
pce/cbpE	putative regulator of pce, sequence specific
pavA	adherence and virulence factor A that binds
cbpD	choline binding protein for cell lysis
SPH_0465	UDP-N-acetylglucosamine 2-epimerase
SPH_0464	Flippase Wzx
SPH_0462	glycosytransferase
SPH_0460	glycosytransferase
SPH_0461	Csp19ag
srtA	pillus associated sortase
srtD	pillus associated sortase
plr/gapA	plasmin receptor - GAPDH
piaA	iron transporter lipoprotein
cppA	C3-degrading proteinase

Table 4.4 List of antibiotics used in detection for antimicrobial resistance gene detection

Antimicrobials included
Aminoglycoside
Beta lactam
Fluoroquinolone
Fosfomycin
Fusidic acid
Glycopeptide
Macrolide
Macrolide-Lincosamide-StreptograminB
Nitromidazole
Phenicol
Rifampicin
Sulphonamide
Teracycline
Trimethoprim

Table 4.5 List of pneumococcal reference genomes used for virulence factor data file for virulence gene detection

***S. pneumoniae* strain**	**Size (bp)**	**Genome reference**
S. pneumoniae CGSP14	2209198 bp	http://www.ncbi.nlm.nih.gov/bioproject/?term=NC_010582
S. pneumoniae D39	2046115 bp	http://www.ncbi.nlm.nih.gov/bioproject/?term=NC_008533
S. pneumoniae Hungary19A-6	2245615 bp	http://www.ncbi.nlm.nih.gov/bioproject/?term=NC_010380
S. pneumoniae R6	2038615 bp	http://www.ncbi.nlm.nih.gov/bioproject/?term=NC_003098
S. pneumoniae TIGR4,	2160837 bp	http://www.ncbi.nlm.nih.gov/bioproject/?term=NC_003028

From 146 pneumococcal genomes from study years 2011/12 to 2012/13, 11 isolates (7.53%) produced positive results for any antibiotic resistance genes (table 4.6). Eight isolates (5.48%) were positive for the *erm*(B)_16 allele, indicating erythromycin resistance. Two isolates (1.36%) were positive for both *mef*(A)_10 and *msr*(D)_2 alleles, indicating macrolide resistance. One isolate (0.68%) was positive for *cat*(pC194), *mef*(A)_10 and *msr*(D)_2 alleles indicating resistance against chloramphenicol, macrolides and telithromycin. Six of the isolates were serotype 15A, 2 were 33F, one was 34, one was 35F and one could not be determined.

Table 4.6 Isolates of *S. pneumoniae* that gave positive results for antibiotic resistance when screened using *in silico* methods and details of co-carriage

Isolate	Resistance Gene	Antibiotic	Co-carriage strain	Serotype	ST
6186	*erm*(B)_16	Erythromycin	*H. influenzae*	15A	63
6200	*mef*(A)_10 *msr*(D)_2	Macrolide	Alone	33F	NF
6332	*erm*(B)_16	Erythromycin	Alone	15A	Indeterminable
7027	*cat*(pC194) *mef*(A)_10 *msr*(D)_2	Chloramphenicol, macrolide, telithromycin	Alone	15A	1591
7041	*erm*(B)_16	Erythromycin	*M. catarrhalis*	15A	63
7093	*erm*(B)_16	Erythromycin	*H. influenzae*	33F	717
7139	*erm*(B)_16	Erythromycin	Alone	15A	63
7140	*erm*(B)_16	Erythromycin	Alone	34	NF
7166	*erm*(B)_16	Erythromycin	Alone	Indeterminable	63
7176	*mef*(A)_10 *msr*(D)_2	Macrolide	*H. influenzae, M. catarrhalis*	35F	446
7217	*erm*(B)_16	Erythromycin	Alone	15A	63

Of the 131 isolates that underwent SRST2 typing to detect a panel of virulence factor alleles derived from the genomes of table 4.5, 124 isolates returned as positive, seven isolates (5.34%) returned results that contained mis-matches (less than 100% match). Of the 124 positive isolates, results gained showed exact

matches (100%) and mis-matches; mis-match results were not included in this analysis. Virulence factor alleles were then matched to the virulence factor gene it encodes (table 4.2), and the results are presented in table 4.7.

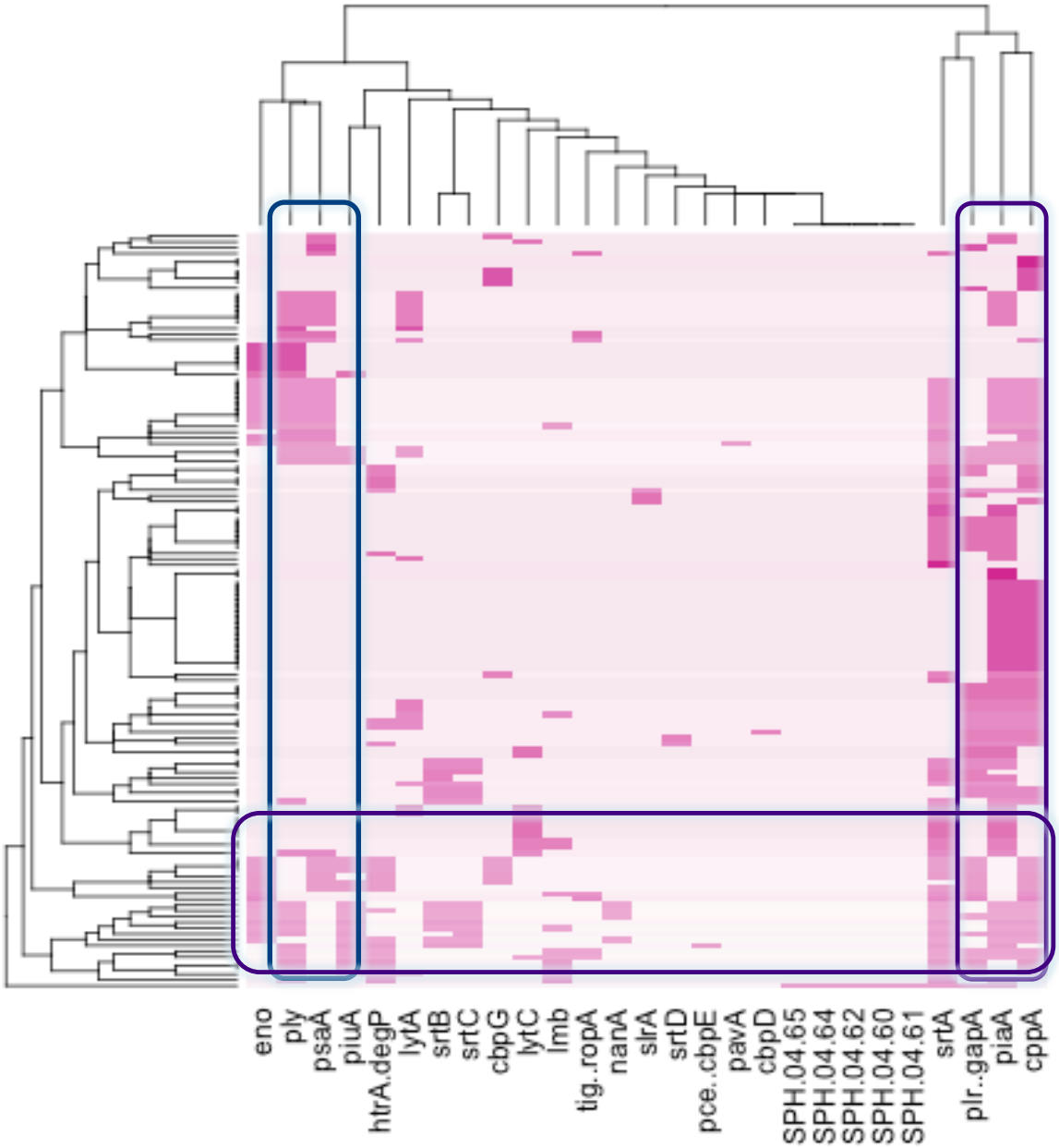
Virulence factor genes present in at least 25% of the population include *pia*A present in 71.76% of the population, *cpp*A present in 61.7% of the population, *srt*A present in 55.73% of the population, *plr*/*gap*A present in 38.93% of the population, *ply* present in 34.35% of the population and *psa*A present in 25.95% of the population. The remaining 21 virulence factors are present in less than 25% of the population. Certain combinations of virulence factors appeared to be specific to both serotype and ST of the isolate. Some serotypes had uniform virulence factors present including; serotype 11A is positive for *ply*, *pia*A, *psa*A, *cpp*A and *srt*A, 15B is positive for *pia*A, *cpp*A, *srtA*/B/C, *cpp*A and *plr*/*gap*A, 23B is positive for *pia*A and *cpp*A, 15A is positive for *cpp*A, *srt*A and *eno*, 35F is positive for *ply* and *eno*, 23A is positive for *pia* and *cpp*A, 35B is positive for *pia*A, *srt*A and *lyt*C, 10A is positive for *pia*A, *lytA*, *ply* and *psa*A, 16F is positive for *lytA*, *ply*, *pia*A*, piu*A, *psa*A, *cpp*A, *srt*A and *plr*/*gap*A and 38 is positive for *cpp*A, *eno*, *srt*A, *plr*/*gap*A and *ti*g/*rop*A. The other 11 serotypes that were detected did not appear to have uniform virulence factors, either due to low numbers of these serotypes or through multiple STs with differing virulence factors.

Figure 4.5 is of a heat-map where the isolates have been mapped together by the panel of virulence factors for which they are positive, two vertical outlines highlight the six most common virulence factors. The horizontal outline on figure 4.5 highlights the isolates that contain the greatest numbers of virulence factors and are comprised of serotype 11A, serogroup 15, serotypes 16F, 19A, 23B and 24F.

Table 4.7 Numbers and percentages of isolates found to be positive for each virulence factor

Gene	Positive (number)	Positive (percentage)
eno	32	24.43
ply	45	34.35
psaA	34	25.95
piuA	22	16.79
htrA/degP	24	18.32
lytA	20	15.27
srtB	15	11.45
srtC	15	11.45
cbpG	10	7.63
lytC	13	9.92
lmb	12	9.16
tig/ropA	7	5.34
nanA	4	3.05
slrA	3	2.29
srtD	2	1.53
pce/cbpE	1	0.76
pavA	1	0.76
cbpD	1	0.76
SPH_0465	1	0.76
SPH_0464	1	0.76
SPH_0462	1	0.76
SPH_0460	1	0.76
SPH_0461	1	0.76
srtA	73	55.73
plr/gapA	51	38.93
piaA	94	71.76
cppA	80	61.7

FIGURE 4.5 HEAT-MAP OF VIRULENCE FACTORS ASSOCIATED WITH INVASIVE DISEASE PRESENT IN CARRIED STRAINS OF *S. PNEUMONIAE* (2011/12-2012/13)



R-studio heat map based on the presence/absence of virulence factors in the form of a similarity matrix using Euclidian clustering. Positive results are marked by dark pink rectangles, X-axis is a list of virulence factors and Y-axis is the isolates. Clustered by isolate and clustered by the genes each isolate contains as well as clustering by gene and the co-presence of other genes. The two vertical rectangles denote the 6 most prevalent virulence factors. The horizontal rectangle denotes isolates that were positive for the most virulence factors.

Table 4.8 Isolates of *S. pneumoniae* of serotype 15A ST63, in depth virulence factor analysis

gene	7041 coverage	6186 coverage	7041 diffs	6186 diffs
*cbpD*_152	100.0	100.0	5snp	5snp
*cbpG*_335	100.0	100.0.		
*cppA*_400	100.0	100.0		
*cps2D*_433	100.0	100.0	5snp1indel	5snp
*cps2E*_147	99.9	100.0	25snp1indel	26snp
*eno*_159	100.0	100.0		
*htrA/degP*_201	100.0	100.0		
*hysA*_40	100.0	100.0	22snp	22snp
*iga*_10	91.2	100.0	208snp2indel501holes	4snp
*lmb*_316	100.0	**Not present**	4snp	
*lytA*_302	100.0	100.0	1snp	1snp
*lytA*_303	93.5	**Not present**	99snp8indel58holes	
*lytB*_69	100.0	100.0	3snp	6snp
*lytB*_77	100.0	100.0	12snp	15snp
*lytC*_119	100.0	100.0	2snp	2snp
*nanA*_42	100.0	100.0	23snp	23snp
*pavA*_98	100.0	100.0	4snp	4snp
*pce/cbpE*_86	95.4	95.5	43snp1indel88holes	45snp1indel85holes
*piaA*_265	100.0	100.0	4snp	3snp
*piuA*_298	100.0	100.0		
*plr/gapA*_247	100.0	100.0		
*ply*_136	100.0	100.0	2snp	2snp
*psaA*_312	100.0	100.0		
*pspC/cbpA*_76	91.4	91.0	145snp7indel175holes	145snp7indel183holes
*slrA*_375	100.0	100.0	3snp	3snp
SP_0347_408	100.0	100.0	15snp	15snp
*srtA*_397	100.0	100.0	1snp	
*tig/ropA*_165	100.0	100.0	7snp	7snp
*wchN*_350	96.6	94.9	105snp6indel26holes	102snp6indel40holes
*wzg*_127	99.9	100.0	11snp1indel	12snp
*zmpC*_16	100.0	100.0	2snp	3snp

Table details gene alleles detected in two isolates of *S. pneumoniae*, both are serotype 15A and ST12. Details include allele names, matching coverage between the isolate and the reference isolate from which the gene allele was derived and the differences between the isolate allele and reference allele.

Virulence factors that resulted in a perfect match between the isolate and reference strain were characterized in Figure 4.5 through the use of a heat-map. For further analysis, two strains of *S. pneumoniae* were chosen from each study time-point (7041 - 2011/12 and 6186 - 2012/13), both isolates were serotype 15A and ST63. Serotype 15A was the most prevalent serotype in the study time-points and was shown to be increasing over the course of the study (Figure 4.1 a-d), additionally; ST63 was associated with antibiotic resistance (Table 4.6). Thirty-one alleles were associated with isolate 7041 with matches of 91.2% to 100%, 29 of these alleles were associated with isolate 6186 with matches of 91% to 100% (Table 4.8). Virulence factor detected include *cbpD* and *cbpG* alleles of choline binding proteins involved in cell lysis, *srtA*/*B*/*C* alleles which encode for pillus associated sortase enzymes. Also present are two important alleles for pneumococcal infection, *lytA* and *ply*. The array of virulence factors detected in carried isolates of *S. pneumoniae*, indicate a strong possibility to cause infection. As the two isolates are of the same ST and serotype from two different time-points, the virulence factor composition remains the same with the exception of two alleles that were not present in isolate 6186 (2011/12) but were present in isolate 7041 (2012/13), *lmb*, an adhesion lipoprotein and the autolysin *lytA*_303 allele.

4.4 DISCUSSION

The carriage study began in 2006/07 when PCV7 and PCV13 VTs dominated pneumococcal carriage but by 2012/13, carriage of PCV VTs is no longer detectable with the exception of one count of 6A, and carriage is almost entirely made up of PCV13 NVTs. The single count of 6A remaining in carriage during 2012/13 may be due to a number of factors, firstly the effectiveness of the PCV13 vaccine was never formally tested but efficacy was derived pre-licensure using data compiled from PCV7 data (Centers for Disease and Prevention, 2010). Although post-licensure studies are underway (Andrews et al., 2014), serotype-specific immunogenicity has been hard to measure due to the high numbers required in a study-set presenting with invasive disease (Black et al., 2000). 6A remaining in carriage may also be due to the study detection limits, 6A may not be the only serotype still in carriage but due to our cohort size and 6As higher prevalence, it is the only serotype we can still detect.

This study has shown that PCV7 and PCV13 VT carriage has decreased and been replaced by PCV13 NVT carriage. No changes have occurred to the level of overall pneumococcal carriage, which remained consistent over a seven-year period. If vaccine replacement has occurred in carriage, then it may also occur for invasive disease, ie PCV7 vaccine replacement was followed by an increase in invasive disease being caused by PCV7 NVTs (Lamb et al., 2014, Chapman et al., 2013, Miller et al., 2011), hence one of the reasons why PCV13 was introduced, now that PCV13 carriage is being reduced, it is being replaced by carriage of PCV13 NVTs (Bottomley et al., 2013) and the same is occurring with invasive disease (Steens et al., 2013), in this body of work, it has been shown that the carried pneumococci contain many virulence factors that were derived from invasive strains, many being a complete genetic match. In the two most

recent years 2011/12 and 2012/13, serotypes 11A, 15A, 15B, 23B and 35B are prevalent or rising, the presence of virulence factors in carriage, and particularly with the most prevalent serotypes of this study, the propensity to cause disease would exist.

Nasopharyngeal dynamics have been explored in other studies focusing on bacterial interactions in the PCV era, general carriage of the individual bacteria has been monitored, there has been less studies focusing on the bacteria that is co-carried with *S. pneumoniae* and if there have been any indirect effects of the PCV vaccine on this carriage. One such study recently reviewed carriage of *S. pneumoniae* and *S. aureus* recently in a range of age groups of older children (aged 4 to 14), and found pneumococcal carriage to be associated with the younger aged participants and also to be inversely associated with *S. aureus*, but co-carriage of both species was very low (around 1%) (Boada et al., 2015). The reality of this study is that it was originally designed to detect a 50% reduction in pneumococcal carriage; the detection of other species of bacteria has been added as the study and its analysis developed. With the hierarchy of bacterial carriage (discussed in Chapter 3) being *S. pneumoniae* and *M. catarrhalis* being the most prevalent species detected, followed by *H. influenzae*, *S. aureus* and non-pneumococcal AHS, co –carriage between *S. pneumoniae* and these species show the same relationship hierarchy. As far as it is possible to say within the scope of this study, no relationships has been seen between *S. pneumoniae* and co-carriage with any of the species included for detection in this study. However this is not to say that a relationship wouldn't be seen using different statistical testing or a larger cohort of isolates or even if there is a relationship at the level of serotype/genotype. Using 95% confidence intervals as markers of statistical significant is a robust way to detect true change, but as the study wasn't designed

for this analysis involving co-carriage, then it may be too stringent to detect subtle change, particularly with low numbers. We can see that study year 2010/11 Produced the greatest percentage of bacterial carriage of all species and, 2009/10 and 2011/12 had the lowest percentages of bacterial carriage of all species. For the study year 2012/13 there were high percentages of carriage for *S. pneumoniae, M.catarrhalis* and *H. influenzae* only and no carriage of AHS or *S. aureus* bar one count. This is an observation made, as this can't be shown currently using the existing statistical analysis. Another way to improve upon this detection of co-carriage would be to collect a greater number of swabs per a year or to group the existing data together as pre-PCV13 and post-PCV13; however this does mask subtle changes if they were occurring during a different time-period and were unrelated to PCV implementation.

MLST data gained from SRST2 methodology produces results that are as accurate as traditional MLST typing. MLST data has been determined for isolates from study years 2011/12 and 2012/13. The study sample is made up of 38 distinct STs and 10 novel allelic combinations termed NF. Of the 20 individual serotypes detected during those two study-years, each distinct serotype was associated with up to 6 different STs. In terms of ST abundance and distribution, no remarkable changes were detected in the two study years. It is important to note that ST data from both these study years were analysed as one data-set as the two year time-period post-PCV13 (without ST data pre-PCV13) does not produce enough numbers to detect change, also ST data from further study years would be required. Certain STs relate with one serotype only whilst others can be seen in several serotypes, such as ST199 seen in both 19A and 15C. To compare the findings in this study, ST distribution of carried pneumococci with published literature is difficult, as many studies have looked at STs of *S.*

pneumoniae but usually in the cases of invasive isolates (Jefferies et al., 2014, Ramos et al., 2014, Inverarity et al., 2011, Jefferies et al., 2004). However some studies published have focused on analyzing ST replacement in the PCV era and found similar results of no difference in ST composition of carried isolates from the early PCV7 era (early 2000s) with the exception of an ST emergence of an antibiotic resistant clone (Hanage et al., 2011). In Canada, ST analysis has been carried out post-PCV13 to detect and characterize pneumococcal profiles due to PCV13 NVT replacement; results indicate that certain serotypes are increasing due to ST expansion at a national and international level (Golden et al., 2015).

Penicillin resistance was not characterized phenotypically or from sequence data for this study due to the difficulty in determining the mosaic of genes that contribute to penicillin resistance, which is a limitation as penicillin's are frequently administered for suspected pneumococcal infections. However, detecting resistance to antimicrobials that does not involve penicillin is an easy process through the use of SRST2 but it is difficult to determine if these isolates are expressing antibiotic resistance without the use of bench lab work, live cultures and direct antimicrobial therapy.

Erythromycin resistance determinants were detected in the isolates (2011/12 to 2012/13) as well as macrolide resistance and resistance to chloramphenicol and telithromycin. Overall, genes encoding resistance to these antimicrobials were detected in around 7.5% of the tested population of isolates through using the exact-match option. The studies discussed above indicate antibiotic resistance to be associated with a particular ST and that antimicrobial treatment can drive resistance upwards by selecting for resistance associated STs and reducing general ST diversity (Keenan et al., 2015). In this study, four pneumococcal serotypes 15A, 33F,

34 and 35F (and one indeterminable type) and four STs, ST63, ST1591, ST717 and ST446 (and one ST that has not been confirmed, and one isolate that did not undergo sequencing), were associated with this resistance. Isolates that were serotype 15A and ST63, were detected in 4 of the 11 cases of resistance and one isolate was found to be serotype 15A but was ST1591. ST63, is associated with 4 different serotypes, 15A, 14, 19A and 19F and the new ST, ST1591 is associated with serogroup 15 and 19F (data gained from www.spneumoniae.mlst.net based on curated data submissions). ST63 associating with serotypes other than the PCV VT serotype 19F is believed to be through a capsular switch (Frazao et al., 2013, Ardanuy et al., 2014) and is associated with invasive disease (Antonio et al., 2009, Sanz et al., 2011).

Virulence factor detection was carried out using around 300 alleles derived from the genomes of five different pneumococcal strains, the results gained show that the most prevalent serotypes contain the most virulence factors as identified using exact DNA sequence matches to the gene fragments from the reference genomes. Four virulent strains and one avirulent strain were used and the results gained show that positive isolates for the various virulence factors match the gene fragment of the reference exactly. This provides an explanation of why the traditional genes, such as *ply* and *lytA*, which can be used in *S. pneumoniae* speciation (Messmer et al., 2004), are not in most of the carriage isolates using this technique (see Figure 4.5). However isolates that were positive for exactly matched DNA sequences of these virulence factors can provide information regarding the distribution of virulence factors among VT and non-VT pneumococci; for example, the most prevalent serotypes post-PCV13 (15A, 23B, 15B, 35B) contain the most virulence factors using this analysis. The implication here could lie with vaccine type

replacement being mediated by NVT serotypes that are able to ‘pick up’ virulence factors to be better at adherence, persistence and ultimately infection.

Using the mismatch gene information from this data set would have produced a very large set of results for all of the isolates that would have to be verified individually, so the full data was used for a sub-set of isolates, two 15A isolates, both ST63 to characterize their virulence profile based on the strong antibiotic resistance profile they displayed and also due to how they are most likely derived from serotype 19F by a capsule switching/recombination event. In depth virulence factor typing indicated propensity to cause disease by encoding virulence factors involved in cell lysis, adhesion lipoproteins, autolysins, pneumolysins and iron transporters, these are mechanisms used for pneumococcal adherence, invasion and persistence.

Several methodologies and routes could have been employed for this virulence factor work, but when working with a pneumococcal carriage data set, it is difficult to gain information beyond basic typing without being overwhelmed with the amount of unknown and unverified information that comes from working with a data set that isn’t classically ‘disease’. For example investigating just a sub-set of genes in a more in-depth manner will allow more specific typing, *ply* and *lytA* genes are an example of this, but not provide comparison to virulent strains.

For future work on this topic, it would be useful to create a database of known genes that assist a strain to invade and cause infection in a host including those from carriage to be used with the SRST2 method, even expanding the number of genomes used in the reference genome database would produce more in depth results and add credibility to the results seen. Additionally, further bioinformatics work can be used to identify genes using the mismatched results produced to identify present genes that just differ in sequence composition from the reference genome.

Summary:

PCV implementation has coincided with a reduction in the carriage of VT serotypes, reductions in pneumococcal carriage was not detected due to increases in PCV13 NVTS. PCV13 NVTs that are prevalent in the era following PCV13 vaccination are potential candidates of vaccine replacement and causing invasive disease in the future due to increased virulence and increased antibiotic resistance as seen with serotype 15A. These serotypes, if proven to cause invasive disease and continue to remain prevalent in carriage will be the focus for future PCV formulations. If we learn to understand virulence factor profiles in carriage isolates, rather than just disease-causing isolates, then comparative genomics will be a useful tool in determining novel vaccine candidates.

5. CARRIAGE DESCRIPTION OF *H. INFLUENZAE* IN THE NASOPHARYNX OF YOUNG CHILDREN DURING THE IMPLEMENTATION OF PCVS IN THE UK

5.1 INTRODUCTION

H. influenzae, is known to be a highly transformable bacterium (Stuy, 1962). An increase in incidence of non-typeable *H. influenzae* (NTHi) has been associated with the observed decrease of *S. pneumoniae* PCV7 VTs as seen in children with otitis media (Wiertsema et al., 2011, Revai et al., 2006, Xu et al., 2012a).

Capsulated *H. influenzae* isolates are assigned a serotype; a-f, depending on their capsular antigens. This leaves a vast population of *H. influenzae* that is un-typed due to either lack of expressed capsule, presence of a capsule not detected by the existing serotyping antibodies, lack of capsule genes or presence of capsular genes unable to be detected using the standard serotype PCR primers. The genetic diversity and similarity within NTHi populations has been explored by multi-locus sequence typing (MLST) where isolates that are identified as NTHi phenotypically, are assigned sequence-types (ST).

Isolates of *H. influenzae* detected from nasopharyngeal swabs in children less than 5 years of age during five annual swabbing time-periods from 2008/09 to 2012/13 has been utilised to characterize *H. influenzae* carriage rates. Methods were expanded upon to determine sequence types, antibiotic resistance genes and virulence factors present.

5.2 OBJECTIVES

- Determine carriage of *H. influenzae* over five year period (2008/09 to 2012/13)
- Determine total genotype carriage through traditional MLST and NGS methodologies of *H. influenzae* over the five year period
- Determine antibiotic resistance genes and virulence factors of carried *H. influenzae* and determine if they are associated with MLST type

5.3. RESULTS

5.3.1 CARRIAGE OF *H. INFLUENZAE* OVER A FIVE YEAR PERIOD

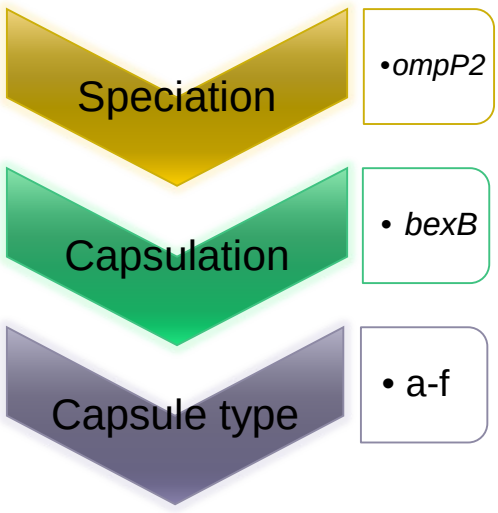
A total of 312 *H. influenzae* isolates were detected over the five swabbing time-points resulting in an average number of 62 isolates collected per a time-point with an average total population carriage rate for *H. influenzae* of 20.3%. The number of isolates that were analysed in this chapter is shown in figure 5.1.

FIGURE 5.1 NUMBERS OF *H. INFLUENZAE* ISOLATES DETECTED OVER FIVE TIME-POINTS AND THE NUMBERS OF ISOLATES USED IN SUBSEQUENT ANALYSES (2008/09 – 2012/13)

Year	2008/09	2009/10	2010/11	2011/12	2012/13
Population total	60	70	69	62	51
OmpP2 lab detection	57	63	66	62	NA
DNA sequencing	60	69	69	57	51

This figure details the numbers of strains of *H. influenzae* detected in each swabbing time point. The methods for analysis are listed stepwise (grey boxes, left hand side), and the corresponding numbers are shown (right hand side of grey boxes). Detection and sequencing numbers that vary from the detected number was due to being unable to extract sufficient DNA from that isolate due to culture/growth issues.

FIGURE 5.2 STEP-WISE PROCESS AND CORRESPONDING GENE TARGET TO DETERMINE CAPSULE-PRESENCE AND CAPSULE-TYPE OF *H. INFLUENZAE* ISOLATES



Isolates of *H. influenzae* underwent PCR (2008/09 – 2010/11) and *in silico* PCR (2008/09 – 2012/13) to speciate and determine capsule type, if present using the above markers.

A combination of wet-lab PCR and *in silico* methods was used to confirm if an isolate was *H. influenzae* and whether it encoded capsule and which capsule type this was (figure 5.2). Analysis was carried out on isolates from 2008/09 to 2011/12 to amplify and detect the presence of the *ompP2* gene, all isolates tested (264), two isolates were negative for the ompP2 gene and 262 were positive for the *ompP2* gene by PCR (2008/09 to 2011/12). The *bexB* gene was used to define if an isolate contained a capsule and if present, the isolate was tested for the presence of capsule specific genes a-f. Isolates in 2008/09 and 2009/10 were tested by standard PCR. Isolates (2008/09 and 2009/10) were tested for the presence of the *bexB* gene to detect if a capsule is present. From 130 isolates tested, five were positive for the *bexB* gene (3.8%). These five genes were tested by PCR for capsule types a to f,

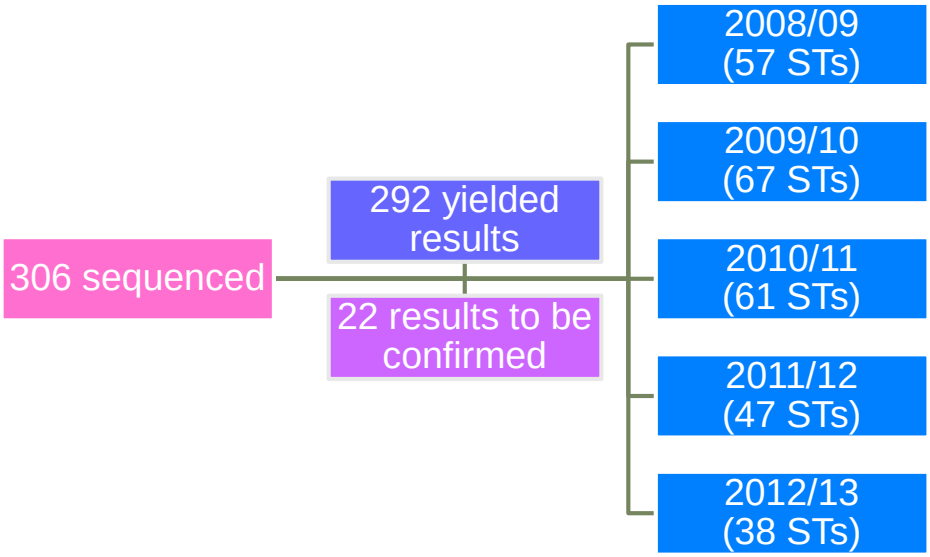
four isolates were positive for capsule type e (3%) and one isolate was positive for capsule type f (0.8%).

245 isolates from study years 2008/09 to 2012/13 were analysed using *in silico* PCR methods. Nine of these isolates were found to be *ompP2* negative (3.7%) and a further two isolates in 2010/11 were detected as being *bexB* positive (0.8%).

5.3.2 CARRIAGE OF *H. INFLUENZAE* GENOTYPES AS DETERMINED BY MLST

The *H. influenzae* isolates found in this carriage study predominately comprised NTHi strains, to elucidate further typing of this data set, MLST typing was used to assign STs to the isolates. There were 306 isolates sequenced (2008/09 – 2012/13) and MLST results were returned for 292 of these isolate, 14 sequenced isolates did not return an MLST result. Of the 292 MLST results, 22 of these remain to be confirmed as they produced a novel allelic combination or novel allelic result (figure 5.3).

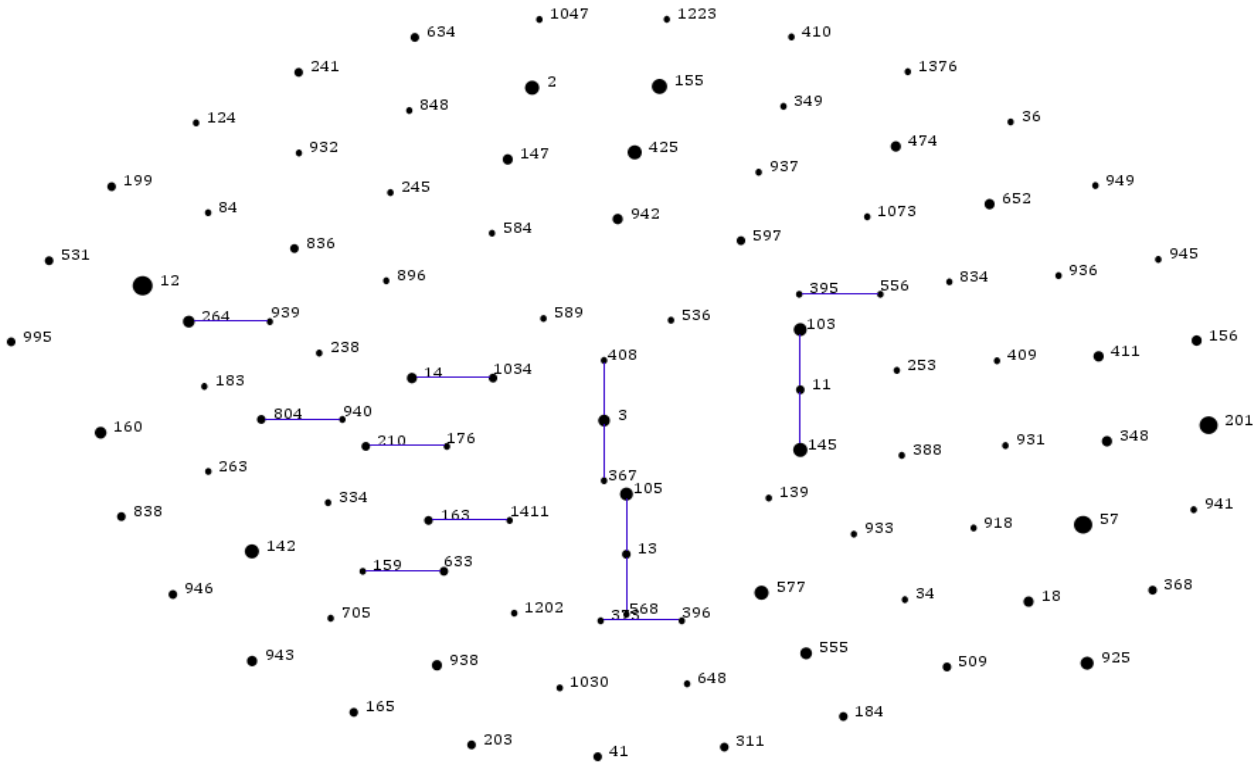
FIGURE 5.3 EXPLANATION OF ISOLATE NUMBERS USED FOR DNA SEQUENCING AND THE RESULTS YIELDED PER A STUDY-YEAR



Total isolate number sequenced (pink box), number of returned MLST result (dark purple box), number of MLST results requiring confirmation (light purple box) and number of ST results per study period (blue boxes).

There were 270 STs assigned and 110 of these STs were distinct, groups were assigned if 6 out of the 7 loci were identical, 11 sub-groups were formed based on this criteria and a 12th group comprising of distinct singletons was also formed (figure 5.4). Three STs were assigned as predicted founders for subsequent STs, ST3 was classed as the predicted founder of both ST408 and ST367, ST13 was classed as the predicted founder of both ST105 and ST568 and ST11 was classed as the predicted founder of both ST 103 and ST145.

FIGURE 5.4 EBURST ANALYSIS OF 270 STS OF *H. INFLUENZAE* (2008/09 – 2012/13)



Of the 270 ST results, 110 distinct STs are marked above by black circles; circle size denotes the number of isolates that were assigned that ST. Purple lines between STs indicate that there are six shared alleles between different STs and hence indicates genetic relatedness. Where there are 3 STs joined by a purple line, the ST in the centre is the predicted founder of that sub-group.

5.3.3 ANTIBIOTIC RESISTANCE GENES AND VIRULENCE FACTORS OF *H. INFLUENZAE* ISOLATES (2008/09 – 2012/13)

Detection of antibiotic resistance genes and virulence factors was carried out using SRST2 as described in Chapter 4 except using a virulence factor database (VFDB - http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Haemophilus/) for *Haemophilus spp*.

From the 306 isolates tested *in silico* for the presence of antibiotic genes, 39 (12.7%) isolates returned a positive result for an antibiotic gene. Of these isolates, 38 (12.4%) returned a positive result for the beta lactamase producing *blaTEM* gene and one isolate (0.3%) returned a positive result for the *blaROB* gene. Of these 39 positive isolates, eight also gave positive results for multiple resistance genes. The aminoglycoside resistant gene *aph3* was present in six of these positive isolates (2%) in the form of the *aph3Ic* allele, three isolates (1%) were positive for the strA4 allele, a further three isolates (1%) for the *strA1* allele and five of these six (1.6%) isolates were additionally positive for the *strB1* allele. *strA* and *strB* alleles encode the str gene which confers aminoglycoside resistance. Sulphamethoxazole resistant genes were detected in three isolates (1%) returning positive results for the sul2 gene and one isolate (0.3%) gave a positive return for the *tetB* gene encoding tetracyclin resistance. Isolates with multiple resistance genes were more prevalent in study years 2009/10 and 2012/13.

Table 5.1 Antimicrobial resistance genes present in *H. influenzae* isolates (2008/09 – 2012/13)

Isolate	Resistant gene	ST
3045	blaTEM-1	577
3068	aph(3')-Ic, blaTEM-1 , strA4, strB1	932
3195	blaTEM-1	103
3207	blaTEM-1	577
3224	blaTEM-1	160
4110	aph(3')-Ic, blaTEM-1 , strA4, strB1, sul2_9	264
4138	aph(3')-Ic, blaTEM-1 , strA4, strB1, sul2_9	-
4140	blaTEM-1	577
4157	aph(3')-Ic, blaTEM-1 , strA1*, strB1	264
4246	blaROB-1 *	-
4267	blaTEM-1	577
4335	blaTEM-1	57
4368	blaTEM-1	-
4379	blaTEM-1	-
4380	blaTEM-1	-
5113	blaTEM-1	-
5134	blaTEM-1	160
5142	blaTEM-1	18
5175	blaTEM-1	245
5184	blaTEM-95*?	NF*
5206	blaTEM-1	160
5224	blaTEM-1	-
5229	blaTEM-1	-
5284	blaTEM-1	-
6054	blaTEM-1	103
6186	blaTEM-1	103
6219	blaTEM-1	577
6240	blaTEM-1	NF*
6315	blaTEM-1	388
7047	blaTEM-1	165
7068	blaTEM-1_5, strA1*, sul2_9, tet(B)_3	3
7093	blaTEM-1	165
7149	aph(3')-Ic, blaTEM-1 , strA1, strB1	142
7151	aph(3')-Ic*, blaTEM-1	1411
7155	blaTEM-1	103
7167	blaTEM-148*?	165
7178	blaTEM-1	103
7179	blaTEM-1	103
7186	blaTEM-1	184

In silico virulence factor typing using alleles encoding for virulence factors of the *Haemophilus* spp was performed on 306 *H. influenzae* isolates (2008/09 to 2012/13), each isolate produced around 90-100 virulence factor alleles each. The most abundant ST was ST12, present in all of the study time-points, two isolates of ST12 were chosen from study year 2008/09 (isolate 3203) and 2012/13 (isolate 7199), the positive virulence factor alleles returned are displayed in table 5.2. There were 93 virulence factor alleles detected from the two isolates, with percentage matches of 92.2% to 100%. Virulence factor genes detected in both of these isolates include high molecular weight protein genes hmw1B and hmw1C, which are adhesions that mediate attachment to epithelial cells. A gene related to adherence and colonisation, *oapA*, was detected as well as several lipooligosaccharide (LOS) related genes including, *kdtA*, *licA*/*B*/*C*/*D* and *lic2*/*3A*. IgA1 protease was detected, which interferes with host barrier functions of igA antibodies. *OmpP2* virulence genes were present; these are outer membrane proteins and can be involved in evading the host immune system. Several proteins and genes involved in iron uptake and transport were detected and these include hemoglobin binding protein (*hgp*) genes *hgpB*/*C, hitA*/*B*/*C* which encode for ferric-binding protein A, cytoplasmic permease and ATP respectively. *HxuA*/*B*/*C* encodes for iron and heme uptake and was also detected. Transferrin binding protein genes *tbpA*/*B* were also detected and also are involved in iron uptake.

The summary of these virulence factors highlights the ability of *H. influenzae* isolate in carriage and their ability to invade, infect and persist within a host.

Table 5.2 Virulence factors determined for ST 12 isolates, 3203 (2008/09) and 7199 (2012/13)

Gene	3203 coverage	7199 coverage	3203 diffs	7199 diffs
comE/pilQ_75	100.0	100.0		
galE_141	100.0	100.0		
galU_185	100.0	100.0		
gmhA/lpcA_251	92.5	92.2	86snp44holes	85snp1indel45holes
gmhA/lpcA_252	90.3	90.3	73snp4indel54holes	72snp4indel54holes
gmhA/lpcA_253	100.0	99.8	22snp	21snp1indel
hemH_153	99.7	99.8	53snp5indel	51snp4indel
hemM_243	100.0	100.0		
hemN_110	100.0	100.0		
hemR_27	100.0	100.0	1snp	
hemX_96	100.0	100.0		
hemY_82	100.0	100.0		
hgpB_16	98.1	98.4	223snp66indel	234snp57indel
hgpB_17	98.4	98.2	177snp51indel	175snp54indel3holes
hgpC_12	95.9	95.8	179snp6indel125holes	185snp9indel125holes
hgpC_9	94.7	94.3	156snp13indel163hole	155snp25indel163hole
hitA_148	100.0	100.0		
hitB_59	100.0	100.0	12snp	12snp
hitC_120	100.0	100.0		
hmw1B_52	99.9	99.9	47snp1indel	54snp1indel
hmw1C_33	100.0	99.9	25snp	28snp1indel
htrB_167	100.0	99.8	27snp	23snp2indel
hxuA_22	97.1	97.1	257snp85indel	258snp84indel
hxuA_24	97.2	97.4	235snp91indel	242snp84indel
hxuA_25	97.9	97.9	219snp68indel	220snp70indel
hxuB_41	99.8	99.8	53snp3indel	53snp3indel
hxuC_28	98.8	98.3	92snp30indel	106snp40indel
hxuC_29	99.9	100.0	127snp4indel	129snp2indel
iga1_1	97.2	97.2	399snp91indel74holes	398snp89indel78holes
iga1_2	98.9	97.6	378snp78indel	353snp69indel75holes
kdkA_226	100.0	100.0	9snp	9snp
kdsA_193	100.0	100.0		
kdsB_216	100.0	100.0	31snp	31snp
kdtA_85	100.0	100.0	1snp	1snp
kpsF_144	100.0	100.0	64snp	64snp
lex2A_265	90.5	95.4	7snp29holes	14indel

lex2B_223	100.0	100.0	3snp	3snp
lgtA_154	100.0	100.0	16snp	16snp
lgtC_151	100.0	100.0	33snp	31snp
lgtF_217	100.0	100.0		
lic2A_164	99.5	99.5	16snp5indel	16snp5indel
lic2A_178	99.2	98.5	20snp7indel	16snp14indel
lic3A_146	99.9	99.8	3snp2indel	2indel
licA_115	100.0	100.0	7snp	7snp
licB_189	100.0	100.0		
licC_231	100.0	100.0		
licD_207	100.0	100.0		
lpsA_196	100.0	100.0	7snp2indel	6snp2indel
lpt6_47	100.0	100.0		
lpxA_210	100.0	100.0	2snp	2snp
lpxB_107	100.0	100.0		
lpxC_174	100.0	100.0	4snp	4snp
lpxC_175	97.1	97.5	152snp12indel19holes	156snp8indel19holes
lpxD_136	100.0	100.0	18snp	18snp
lpxH_227	100.0	100.0		
lpxK_147	100.0	100.0	41snp	41snp
lsgA_100	100.0	100.0		
lsgB_176	100.0	100.0	24snp	24snp
lsgC_125	100.0	100.0	1snp	1snp
lsgD_212	99.9	99.9	2indel	2indel
lsgE_186	100.0	100.0		
lsgF_203	100.0	100.0	1snp	1snp
manA_268	100.0	99.5	10snp	9snp1indel
manB_48	100.0	100.0		
mrsA/glmM_76	100.0	99.9	43snp	41snp2indel
msbA_38	93.5	92.2	151snp5indel110holes	145snp3indel135holes
msbA_39	100.0	100.0	21snp	21snp
msbB_159	100.0	100.0	1snp	1snp
neuA_236	100.0	100.0		
NTHI1224_54	100.0	100.0	14snp	14snp
oapA_81	100.0	100.0		
*ompP2*_111	93.9	93.9	58snp76indel	57snp79indel
*ompP2*_116	100.0	100.0	2snp2indel	4snp1indel
*ompP2*_118	93.9	94.1	69snp11indel61holes	72snp9indel61holes
*ompP2*_126	99.2	99.3	41snp16indel	44snp13indel
ompP5_117	96.0	96.4	58snp43indel	64snp43indel
opsX/rfaC_129	100.0	100.0	28snp	28snp
orfM_250	100.0	100.0	18snp	18snp
pgi_42	99.9	100.0	71snp1indel	72snp

pilA_262	100.0	100.0		
pilA_263	99.3	99.1	38snp4indel	37snp5indel
pilB_67	100.0	100.0		
pilC_93	100.0	100.0		1snp
pilD_234	100.0	99.9	12snp	11snp1indel
rfaD_171	100.0	100.0	21snp	21snp
rfaE_62	100.0	100.0		
rfaF_132	100.0	100.0	2snp	2snp
rffG_130	100.0	100.0		
tbpA_23	99.5	99.6	77snp21indel	79snp19indel
tbpB_36	97.4	97.8	247snp52indel7 holes	215snp51indel1 holes
waaQ_119	100.0	100.0		
wecA_121	100.0	100.0		
yhbX_58	100.0	100.0	21snp	21snp

5.4 DISCUSSION

Carriage of *H. influenzae* remains consistent over 5-year period, pre PCV13 and post PCV13, similar to what has been detected with other bacterial species in this carriage study. The average carriage rate of *H. influenzae* in this study is 20.3% (range 17.5% to 24% for the period 2008/09-2012/13), a recent study in China gives an average carriage rate of 22.3% over four time-points between 2000 and 2012 in children less than 5 years of age (as per this study) with beta-lactamase resistance averaging 18.8% (Zhu et al., 2015) compared to the 12.7% average of beta-lactamase determinants detected in this study, this could be due to the data set used in the paper being from children with acute respiratory tract infections. Geographical and ethnic differences in *H. influenzae* carriage have been detected with carriage rates for aboriginal and non-aboriginal children in Australia being 36% and 9% respectively (Pickering et al., 2014c), this study also noted that true carriage detected by 16S rRNA sequencing was lower than rates detected by standard culture as the sequencing methods is more sensitive than standard culture. In Kenya, carriage rates of *H. influenzae* were analysed both before (54%) and after (40%) PCV10 implementation (Hammitt et al., 2014) yet both rates are higher than that reported in this study, this is most likely due to several reasons including, geographical, ethnic and cultural differences.

H. influenzae carriage as reported by this study was detected and confirmed with standard culture before being additionally confirmed by PCR of the *ompP2* gene (2008/09 – 2011/12) and through *in silico* PCR of the same gene (2008/09 – 2012/13). The *in silico* data produced a higher number of ompP2 negative results than what was seen by PCR but there may be issues involved in sequencing quality

of certain isolates that needs to be investigated before a conclusion can be made on the use of the bioinformatics program versus the wet-lab methodology. NTHi was characterised by a negative *bexB* PCR (2008/09-2009/10) and *in silico* PCR (2008/09 – 2013/13), these methods were comparable and is acceptable as long as sample set does not contain 'fuzzy' isolates/potential *H. haemolyticus*, particularly non-haemolytic *H. haemolyticus*. Detection of non haemolytic *H. haemolyticus* was not accounted for due to the disparity in the methods required for detection (Pickering et al., 2014b), currently sensitivity for the most discriminatory method for differentiation between these two species is 92% (Pickering et al., 2014a) but can be increased to 99.6% by using specialist equipment (Bruin et al., 2014). Discrimination between these two species may marginally reduce the carriage rate of *H. influenzae* reported by this study (Fenger et al., 2012).

The high prevalence of NTHi presents an issue within itself, it is difficult to characterize relatedness of isolates 'with ease', any species that does not have a capsule and presents as being genetically diverse, so in this case MLST was used to assign sequence types and characterize genetic relatedness of an isolate based on ST. Method evaluation between MLST by traditional methods and *in silico* MLST was compared for study years 2008/09 and 2009/10 and there was 100% concordance between the two methods,

H. influenzae, particularly NTHi are genetically diverse and very little information can be inferred from MLST as few isolates form clonal complexes due to the high rates of genetic diversity, therefore there are more singletons in a population and isolates are reported as being genetically diverse. This study identified three clonal complexes in the population of 270 isolates over all time points (2008/09 – 2012/13), CC3, CC11 and CC13, these three CCs cannot be considered as distinct

or used as a measure of relatedness to other *H. influenzae* strains as other studies have shown that even commensal *H. influenzae* strains and those isolated from disease can cluster together (LaCross et al., 2013). Although there is very little research carried out on the genetic relatedness of NTHi STs, two studies report that predominating STs have not been detected in either carriage/disease (Bertelle-Ibrahim et al., 2013) or beta lactamase producing strains (Kaur et al., 2011). However other studies have reported associations of *H. influenzae* STs between children and their primary care giver, indicating a transmission pattern (Schumacher et al., 2012), associations between STs of strains detected from two different sites in the same individual, middle ear and nasopharynx of children with otitis media, have also been reported (Langereis et al., 2013), finally it has been proposed that STs with a *ftsI* mutation strongly relates to beta lactam resistance with an altered penicillin binding protein (Skaare et al., 2014).

Antibiotic resistance of *H. influenzae* was present in just over 12% of strains in the dataset through detection of blaTEM genes, resistance of other antibiotics present and multiple resistance markers in some strains. Beta lactam resistance has been encountered previously in other studies (Garcia-Cobos et al., 2014) and increased beta lactam resistance has been linked to clonal expansion of strains (Fleury et al., 2014). However with results indicating that at least one child in every eight children is carrying some form of antibiotic resistant *H. influenzae*, there is cause for concern especially given the ability of *H. influenzae* to undergo recombination (Van Eldere et al., 2014).

Invasive *H. influenzae* disease has reduced since Hib vaccine implementation, among the reduced numbers of invasive disease cases still caused

by *H. influenzae*, NTHi is the most prevalent cause of these infections (Wan Sai Cheong et al., 2015), particularly in the immunocompromised (Gkentzi et al., 2012). NTHi is a cause of non-invasive infections including otitis media, conjunctivitis and COPD, once again, the strains of NTHi causing these infections have been described as diverse (Puig et al., 2014). With therapeutic treatments limited to antibiotics, for which resistance is present, other forms of treatment need to be explored. A preventative measure to NTHi infections would be preferable; a vaccine that reduces colonisation primarily and also infection and persistence is becoming a requirement. Difficulties arise as NTHi is unencapsulated, but as shown in this study, encodes a wide variety of potential virulence factors that also show consistency between strains. For a virulence factor to be used as a vaccine candidate it needs to be present ubiquitously in all NTHi strains, it also needs to be immunogenic in the human host, meaning that such molecules often play a vital role in NTHi colonisation and infection. Studies are now underway to characterise virulence factors of NTHi and the role they play in infection in the host, this is to determine suitability as a vaccine candidate. It has been reported that there are differences in virulence amongst NTHi strains (Melhus et al., 1998), with higher prevalence rate of adhesin genes playing a role in disease causing strains (otitis media) versus a higher prevalence of heamagglutinating pili genes in strains from carriage (Ecevit et al., 2004). Lipooligopolysaccharides (LOS) have been discussed as being potential vaccine candidates, early studies show that a LOS based vaccine can reduce NTHi infection in otitis media in human trials (Gu et al., 1997). Anti-NTHi LOS antibodies have been reported to be naturally occurring in individuals already (Choi et al., 2015), but the developed vaccine has had formulation changes made to it since it was first reported with varying results ranging from an increase in antibody levels of human subjects post immunisation (Gu et al., 2003) and clearing NTHi carriage in chinchilla models (Hong et al., 2010) but no further work has been presented on this as a potential vaccine for NTHi. Recently the outer membrane composition of *H.*

influenzae has been investigated and several components identified as being potential vaccine candidates including *lytM* involved in NTHi pathogenesis (Ercoli et al., 2015), outer membrane vesicles (OMV) (Roier et al., 2014) and the adhesin Hia (Atack et al., 2015).

Virulence factors identified in this study of two NTHi strains of ST12 identify genes related to adherence, colonisation, LOS related gene, outer membrane protein genes, pilus genes, haem binding and iron transporter genes, similar results to the work that is currently being carried out by other groups in the field (Morey et al., 2013, Harrison et al., 2013, Mason et al., 2011, Mistry and Stockley, 2011, Novotny et al., 2009). The advantage to the study presented in this thesis is through the methodology of SRST2 used for detecting known or proposed virulence factors. SRST2 allows a script to be executed using a compiled database and returns all proposed matches. This allows a high volume of sequenced strains to be analysed in a non-discriminatory manner. This study and the follow up work will be the first time a large number of carried isolates of *H. influenzae* have been typed, sequenced and analysed using bioinformatics to determine all known virulence factors within a carriage population and have STs directly compared to each other. Virulence factors that are detected in all strains could be taken forward and their role in colonisation and infection explored. This work is the basis towards identifying virulence factors present in a large and diverse population

Summary:

Carriage of *H. influenzae* is predominately made up of NTHi; ST typing indicates these NTHi isolates are part of a genetically diverse population. Antibiotic resistance gene detection determined beta lactamase resistance to be the most common source of antibiotic resistance with more than 1 in 8 strains to display beta

lactam resistance. Virulence factor gene detection shows *ompP2* to be ubiquitous in strains and many genes involved in adherence and colonisation as well as iron transporter genes present in the population. Virulence factor profiles also are distinct to an extent depending on the ST of the strain. Further analysis would be required to determine the genes present in all strains for the detection of potential vaccine candidate genes.

6. CHARACTERISING BACTERIA OF THE NASOPHARYYNX USING COMMUNITY PROFILING

6.1 BACKGROUND

Studies have shown the difficulty of relying on standard microbiological culture alone for the accurate identification of bacterial species in routine diagnostic labs, with some species presumed to be under-reported due to culture difficulties (Moter et al., 2010). Studies that focus on bacterial carriage using molecular methods report that a greater diversity of organisms is seen when compared to culture-based results (Power et al., 2005). Some of the reasons for this could be that organisms are difficult to culture, viable but present in low abundance or are genetically similar to another species. This is demonstrated by the level of similarity between *H. influenzae* and non-hemolytic *H. haemolyticus*, two organisms that can only be discriminated by molecular methods(Binks et al., 2012).

16S community profiling is the term given to a variety of methods utilizing the variable nature of the 16S rDNA gene that allow researchers to characterize the microbiota of an environmental niche(Charlson et al., 2010, Hilty et al., 2012). Characterization is achieved through the use of broad-range primers covering a region of the eubacterial 16S rDNA gene. Regions targeted are termed hyper-variable V regions 1 to 9. Each V region allows identification of a eubacterial organism to genus level but some V regions allow further identification so that species identification is possible. It is possible to analyze a large number of samples for sequencing on a “next generation” sequencing platform to gain in-depth community profiles to the level of genus and sometimes species for a collection of bacteria present in a single sample. 16S community profiling is particularly useful in the case where organisms may be difficult to culture in media or may not be present

in large numbers. The ability to process large numbers of samples in a single run greatly reduces costs associated with next-generation sequencing.

6.2 OBJECTIVES

- To characterize the inter- and intra-sample variation of the paediatric nasopharyngeal microbiota in a culture-independent way using 16S community sequencing.
- To determine a viable biological material for use in 16S community sequencing protocols
- Create a protocol adapted to suit the samples available from this project for community sequencing
- Report carriage of *H. influenzae* and *S. pneumoniae*, based on community sequencing bias and compare this with estimates gained by culture.

6.3 RESULTS

The samples used in this work are shown in table 6.1. Samples were chosen to represent sample diversity among our collection of swabs and stored material and to determine whether 16S sequencing could be carried out on several different storage media that contain nasopharyngeal bacterial material. This was to determine which starting material produced results comparable to the sequencing of a nasopharyngeal swab previously frozen and stored in glycerol (a standard process). Samples consisted of: bacterial material that was deposited onto cryogenic beads from a swab, nasopharyngeal swabs, 50% glycerol/BHI that contained a stored swab and a swab after it was vortexed strongly plus controls. It was observed that the bacterial species initially cultured from these samples differed; these culture results were recorded (Table 6.1) and used as a comparator dataset in the analysis.

Table 6.1: Samples and sample materials used in 16s community profiling project.

Patient number	**Sample type**	**Barcoded primer**	**Cultured bacteria**
Control	HPLC grade H_2O	MID-4	…
Negative swab	control	MID-6	No growth observed
Positive swab	control	MID-7	Mixed flora
3189	Cryogenic bead previously frozen at -80°C	MID-8	No growth observed
3294	As above	MID-10	*H. influenzae*
4131	As above	MID-11	*S. pneumoniae*. *H. influenzae*, other-αHS
5015	Nasopharyngeal swab stored in glycerol at -80°C	MID-14	S*. pneumoniae, H. influenzae*, MSSA
5027	Glycerol in 50% BHI, previously frozen at -80°C	MID-15	Not cultured
5027	Nasopharyngeal swab stored in glycerol at -80°C	MID-16	*S. pneumoniae*, *H. influenzae*
5027	swab vortexed into 1.5ml media (5ml BHI/50µl Hemin/1µl β-NAD)	MID-17	Not cultured

6.3.1 PROCESSING OF HYPER-VARIABLE REGION V4 SEQUENCES

16s rDNA gene fragment sequences were obtained using the methods described in section 2.6. Using the online tool MG-RAST (Meyer et al., 2008), sequence files were uploaded to the website where they were analyzed for sequence similarity to a known database named M5NR, a non-redundant protein data-base, (Wilke et al., 2012). This database allows the user to have their sequences matched to similar sequences simultaneously through ten well-known databases containing 16S sequences including SILVA and NCBI. Sequences that had at least a 60% match to a sequence from the M5NR database are included in the below table 6.2. The table shows taxonomy to the genus level as well as abundance of the genus as predicted by the numbers of that particular matching sequence in a sample. Differentiation to the level of species was not achievable for all organisms in the samples due to the chosen region of V4 of the primers so all results herein are discussed at the level of genus.

6.3.2 ACCURACY OF V4 REGION SEQUENCE ASSIGNMENT

The data indicated that *Haemophilus* spp. was detected by 16S sequencing in sample 5027 using the glycerol stock where the swab head was stored and in the swab head when incubated in enriched media but not from the swab head alone or from cryogenic beads (table 6.2). *Streptococcus* spp. was identified by 16S sequencing in sample 5027 in the glycerol stock, the swab head and swab head in enriched media, it was also identified in the positive control (table 6.2), but it was not identified in the samples 4131 or 5015, where it was cultured (tables 6.1 and 6.2). *Staphylococcus* spp. was not detected by 16S sequencing in any of the seven

samples, however only one of the seven samples cultured positively for a *Staphylococcus* spp., *S. aureus* in the sample 5015, swab head (table 6.1).

Through 16S sequencing, the greatest level of diversity in organisms was detected in the glycerol stock 5027; it also correctly identified two of the organisms seen by culture. Bead 4131 had the lowest level of diversity of organisms as detected by 16S sequencing, and despite being culturable for 3 organisms; none of these were detected by 16S sequencing.

The results shown in table 6.2 highlight only the top ten genus matches. Ten results per sample were considered the 'cut-off' point of the results as more matches were produced at very low percentage matches. It is difficult to interpret the results from the study samples due to the results from the negative control swab. The negative control produced a large number of sequence matches suggesting possible contamination of the negative control possibly contaminants from the reagents of the DNA extraction kit. The implication of this is that matches observed in study samples cannot be confidently interpreted as indicating the presence of the genus in the study sample.

Table 6.2: Greatest matching bacterial genus for each sample sequenced and analysed using MG-RAST. Matching sequence percentage shown

Negative swab	**%**	**Positive swab**	**%**	**3189 bead**	**%**
Ralstonia	77.8	Streptococcus	53.6	Ralstonia	75.8
unclassified	5.6	Corynebacterium	50.2	Marinospirillum	5.1
Marinospirillum	5.1	Ralstonia	14.7	Terrimonas	2.9
Caulobacter	3.8	Rothia	2.4	Synechococcus	2.7
Synechococcus	3.5	Mycobacterium	2.0	unclassified	2.5
Terrimonas	3.2	Lactobacillus	1.5	Lactobacillus	2.3
Lactobacillus	2.9	Singularimonas	0.9	Oryza	2.2
Oryza	2.7	Synechococcus	0.9	Pelomonas	2.0
Heliobacterium	2.3	Oryza	0.5	Caulobacter	1.7
Sphingomonas	2.0	Clostridium	0.4	Singularimonas	1.0
3294 bead	**%**	**4131 bead**	**%**	**5015 swab**	**%**
Ralstonia	70.8	Ralstonia	81.2	Ralstonia	56.4
Singularimonas	3.9	Marinospirillum	5.2	Methylophaga	14.0
Synechococcus	3.8	Caulobacter	3.8	Moraxella	12.8
Pelomonas	3.5	Synechococcus	3.5	Marinospirillum	4.3
Oryza	3.2	Lactobacillus	3.1	Synechococcus	2.6

Lactobacillus	3.1	Oryza	2.7	Caulobacter	2.3
unclassified	2.7	Terrimonas	2.7	Sphingomonas	1.7
Terrimonas	2.6	unclassified	1.9	Lactobacillus	1.7
Ruminococcus	2.5	Pelomonas	1.7	Cupriavidus	1.5
Heliobacterium	2.3	Burkholderia	1.2	Oryza	1.5
5027 glycerol	**%**	**5027 swab head**	**%**	**5027 swab vortexed in media**	**%**
Streptococcus	31.1	Ralstonia	52.9	Ralstonia	38.3
Moraxella	24.7	Moraxella	11.3	Moraxella	8.5
Haemophilus	21.5	Streptococcus	8.7	Streptococcus	7.5
Methylophaga	11.6	Methylophaga	7.0	Haemophilus	6.1
Singularimonas	5.7	Singularimonas	4.1	unclassified	3.5
Ralstonia	5.4	Lactobacillus	2.8	Caulobacter	3.3
Terrimonas	3.1	Synechococcus	2.4	Sphingomonas	3.3
Burkholderia	1.4	Actinobacillus	2.1	Synechococcus	2.8
unclassified	1.3	Bradyrhizobium	2.0	Terrimonas	1.6
Gallus	1.2	unclassified	1.9	Heliobacterium	1.4

6.4 DISCUSSION:

6.4.1 16S COMMUNITY PROFILING VALIDITY

This project has used next generation sequencing for characterizing the nasopharyngeal microbiome(Turnbaugh et al., 2007, Bogaert et al., 2011). 16S community profiling appears to provide a list of diverse genera derived from the input DNA. Also I have demonstrated that a glycerol stock containing a swab head is a good alternative starting material for 16S community profiling. It can be considered that the level of contamination seen in the negative control invalidates the results derived, but 16S community profiling does require user input in deciding what a likely contaminant is and what a true result is. Even with bioinformatics' support, each genus result given must be manually studied and a decision should be made whether the genus is likely to be present within the sample, for example, Eukaryotic and Archea genera are likely contaminants in respiratory samples.

This study shows the need for standardization when working with next generation sequencing protocols. The recommended kits/equipment must be fully validated before being used as even DNA extraction kits introduce contamination to the highly sensitive 16S amplification PCR.

Preliminary data suggests that cryogenic beads are not a suitable starting material for 16S community sequencing due to not being able to extract a high quality DNA capable of producing accurate 16S sequences. Glycerol stock that contained a swab head and a swab head in an enrichment media produced the best result, in that they were accurate to the culture results to an extent. However there was the issue of the presence of Ralstonia spp. in the samples, these are contaminants and have been the most abundant result seen in all samples bar the

positive control and the glycerol stock sample. When a contaminant is present in what is called a low-density sample, such as these samples where there are low amounts of bacterial DNA available for amplification, an over-representation of the contaminant is observed. More stringent parameters when searching for sequence matches on databases may help reduce this bias, but ideally analysis should be carried out by bioinformatics and scripting to remove contaminants from the sequence pool and only show true results.

6.5 16S PILOT STUDY LIMITATIONS

Swab storage has been shown to effect DNA quality and therefore 16S community profiling results (Kwambana et al., 2011). It was very difficult to extract concentrated, high quality DNA without resorting to using an enrichment media with incubations and extended vortexing, which may bias the sample. A media enrichment step adds an extra step, with extra manual handling needed with the tube increases exposure, which may increase the potential for contaminations. The low-yield DNA in some samples is most likely an effect from the bacteria kept long-term in frozen-storage, in a sub-optimal storage medium, resulting in degraded DNA (Kwambana et al., 2011). The genus *Streptococcus* was only once correctly identified, which may be an issue with primer-pair specificity or the hyper-variable gene region chosen as the gene region may not be sufficient to identify all organisms at the genus level.

A dedicated space for sample preparation will be used for future work as sample preparation in this study prior to sequencing was carried out in a microbiology laboratory that routinely cultures bacteria and patient samples thus easily introducing contamination to the 16S amplification PCR. In addition, some

prior analysis of reagents may be required as these may be naturally contaminated as some other groups have also found. Use of an ‘extraction kit control’ each time sequencing is performed has been used as a method to counter this problem (personal communication). Another issue encountered during sequencing was a presence of small bands of amplicons or possibly unused primers, which were not detected by the Agilent Bioanalyser prior to a sequencing run, nor were they removed by the PCR clean-up kits used, this may be due to the size ranges these kits are specific to removing and therefore outside of the detection boundaries of the Bioanalyser. This caused one failed run, and Roche had to be contacted to remove these small bands.

6.6 16S PILOT STUDY FUTURE WORK

To expand this project, emphasis needs to be placed on validation of which hyper-variable 16S V regions are optimal for respiratory swab samples, effective DNA extraction and a more stringent view on contamination during PCR. In order to do this it is planned that the methods be improved to incorporate the use of primers with increased target specificity as well as separate pre- and post-PCR rooms for 16S amplification and analysis. Alternative DNA extraction methods (Biesbroek et al., 2012) will also be investigated with the aim of producing improved DNA yield without the use of media which may bias results towards the preferential growth of some species more than others, depending upon the enrichment media used.

The work presented here represents developmental work and will be used as pilot data. Further refinement of this technique will be required to improve on 16S community sequencing for this study. In parallel with this work-stream an additional technique will be explored for its utility in identifying microbial community members in

the study samples. Plex-ID is a piece of equipment that uses a high resolution mass spectrometry that determines the base composition across different gene loci for a broad range of organisms including bacteria, fungi and viruses for screening and identification. The machine uses kits for the identification of broad panels of organisms; the kit you choose is dependent upon the sample being investigated. This technique may be used to compare study samples against a broad reference panel of respiratory organisms to investigate the nasopharyngeal niche (Jacob et al., 2012).

Results of this and improved 16S analyses will be compared with culture results of the carriage study to identify differences between selective culture-based, selective non-culture based and non-selective methodologies (Harris and Hartley, 2003).

7. DISCUSSION

7.1 KEY FINDINGS

Changes in microbial carriage of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus* within the nasopharynx of children less than five years of age were not detected at the species level. No relevant changes in carriage of these bacteria at the species level were detected either year on year for the duration of the study or for carriage when study time-points were grouped into pre and post PCV13 implementation. When determining pneumococcal serotypes although there were no changes in pneumococcal carriage at the species level over the study period 2006/07 to 2012/13, a reduction in the proportion of VT pneumococci were detected. A reduction in both PCV7 and PCV13 VTs, with a corresponding increase in PCV13 NVTs was observed over the duration of the study indicating that PCV implementation has reduced the carriage of VT serotypes.

Detection of known resistance genes within *S. pneumoniae* from 2011/12 and 2012/13 was used to estimate prevalence of resistance to around 14 antibiotics and groups of antibiotics. Erythromycin resistance as determined by detection of alleles of *ermB* was the most prevalent gene detected. Serotype 15A and ST63 isolates displayed the most frequent antibiotic resistance to *ermB* in five of the eleven positive isolates. Additionally a 15A isolate of ST1591 returned positive results for three types of antibiotic resistance. Virulence profiling of *S. pneumoniae* was completed using DNA sequence mapping. Two different matching criteria were employed; 100% matching and 90% matching. Exact DNA sequence matching results for all isolates (2011/12 and 2012/13) indicated that

the most prevalent serotypes contain the most virulence determinants derived from the reference gene panel. Allowing mis-matches enabled increased sensitivity but decreased specificity. Applying this method to two isolates of serotype 15A/ST63, results indicated concurrent results between the virulence factor profiles of both strains. *S. pneumoniae* serotype 15A would appear to be a prevalent isolate involved in PCV13 NVT replacement with the ability to drive *ermB* resistance in the population with the propensity to contribute to disease due to the variety of virulence factors it contains. Whether these mechanisms are due to its capsular type or ST is currently unknown.

H. influenzae carriage predominately consists of NTHi (2008/09 – 2012/13), which have been determined as being genetically diverse according to MLST. *In silico* antibiotic resistance was detected predominately through the presence of the *blaTEM* gene in around 12% of the isolates tested, resistance did not appear to be mediated by a particular set of STs. *In silico* virulence factor typing was performed on all isolates with a large return of present virulence factors for each isolate. In-depth virulence factor typing was performed on two isolates of NTHi, ST12 as they were the most prevalent STs in the population (2008/09 – 2012/13) and were detected in each study year. Virulence factors of interest included those that aid colonisation, adherence and iron transport. There was complete concurrence of virulence factor profiles between both isolates of ST12.

6.2 DISCUSSION OF THESIS RESULTS

Nasopharyngeal co-carriage as reported by this study has shown no changes at the species level either pre or post PCV13, but a reduction in PCV VTs is present with a corresponding increase in PCV13 NVTs. Other studies have reported varied results including a reduction in *S. pneumoniae* PCV7 VT serotypes with a corresponding increase in *H. influenzae,* particularly NTHi (Wiertsema et al., 2011). The eradication of PCV7 VT in the nasopharynx has also been associated with higher rates of *H. influenzae* and *S. aureus* carriage in young children and infants, highlighting those virulent serotypes of *S. pneumoniae* also have a competitive relationship with *S. aureus* as well as *H. influenzae* (Spijkerman et al., 2012, Biesbroek et al., 2014). Despite not detecting changes in microbial carriage at the species level during PCV implementation, incidences of bacterial co-carriage are important to report to inform future vaccine developments. This is important as the effect of vaccines targeting nasopharyngeal pathogens, which reduce or prevent carriage of the target capsular types may produce indirect effects, as the ultimate balance between co-colonising organisms is unknown. The future of vaccination is under scrutiny with each vaccination implemented against pneumococcal specific serotypes or serogroups, as vaccine-type replacement is detected in the years following vaccination.

Antibiotic resistance genes are present in around 10% of *S. pneumoniae* and *H. influenzae*, predominately comprising of *ermB* and *blaTEM* gene presence in the two species respectively. Due to co-carriage of multiple strains, individuals could be harbouring multiple strains of bacteria with multiple types of resistance, potentially acting as reservoirs for other bacterial species and driving resistance. Clonality has been detected in strains of *S. pneumoniae* that confer antibiotic resistance, including

a erythromycin resistant clone of serotype 14 detected in Greece that is similar to a UK serotype 14 resistant strain (Fotopoulou et al., 2003). International resistant strains origination from Taiwan were found to play a considerable role in contributing to antibiotic resistance in China (Geng et al., 2014). Two clones of *S. pneumoniae* have been found to contribute to the distribution of penicillin resistance in the USA, with one clone being detected in 30 of the 39 American states (Corso et al., 1998). Three ST clones of *H. influenzae* were detected in Italy and were associated with almost 50% of *blaTEM* resistance (Giufre et al., 2013). Although a predominant ST wasn't linked with resistance of *H. influenzae* in the data-set of this thesis, it was possible to link serotype 15A/ST63 as a driving factor of antibiotic resistance for *S. pneumoniae*. Serogroup 15 is also an emerging serotype of the latter years of the study and similar results in terms of serotype 15A/ST63 being a significant PCV13 NVT contributing to antibiotic resistance has been detected in a recent study in China (Liyanapathirana et al., 2015). On the theme of serotype, virulence factor analysis indicates that 15A/ST63 possess many genes involved in adherence, colonisation and general virulence. To confirm this as a significant outcome, comparison work with other serotypes and STs would have to be completed first as pneumolysin has been shown to be strain-dependent (Harvey et al., 2014). However, this is still interesting as it places the serotype as an increasing PCV13 NVT contributing towards resistance with the ability to contribute to disease. No particular ST or group of STs could be associated with making a substantial contribution to resistance and enhanced virulence among the *H. influenzae* isolates. This was due to the high ST diversity of the population. However, upon analysis of the virulence factor profile for the most prevalent ST, ST12, we can see that there a large number of genes with the ability to facilitate bacterial colonisation were identified. Genes associated with adherence and transport systems, which can contribute to persistence, were also observed. Researchers are now focusing on determining which of *H. influenzae*'s many virulence factors actually contribute to pathogenesis as NTHi is now

recognized as a pathogen contributing to COPD and otitis media (Ercoli et al., 2015, Harrison et al., 2013, Wong and Akerley, 2012). The co-carriage of both species of bacteria could help facilitate resistance and virulence through sharing the same ecological niche with DNA exchanges made due to their transformable nature as seen between *H. influenzae* and *N. meningitidis* (Gawthorne et al., 2012) and *H. influenzae* and *H. haemolyicus* (Witherden et al., 2014). Furthermore, it would appear that the ST of an isolate rather than its serotype mediates virulence factor presence and that these virulence factor profiles appear to be consistent within isolates of the same ST.

The work carried out in this thesis focuses on bacterial carriage and co-carriage that is often overlooked following vaccine implementation, it also highlights some of the antibiotic resistance genes present in the population and uses an *in silico* method to determine all known virulence factors that aid a bacterium's survival within a host. Virulence factors are often a focus within invasive disease isolates but it has been neglected to determine the presence in carriage strains.

7. FUTURE WORK

Monitoring nasopharyngeal bacterial interactions over PCV13 introduction should be continued along with using epidemiological based statistical analysis that may be better suited to this particular study. Furthermore, inclusion of the questionnaire data could be included which contains vaccine status, recent antibiotic use and age of child in months for more effective stratification of the pneumococcal serotypes detected. The continuation of PCV VT determination should also be continued but with added focus on PCV13 NVT increases and on serotype 15A due to the work presented in this thesis. NTHi carriage monitoring should be continued with further study years with the purpose of creating a larger data-set of NTHi STs to compare distribution year on year. As research continues with the search for an NTHi vaccine, a large database of recent NTHi carriage that has already been sequenced is a valuable commodity for gene comparisons. Virulence factor profiling for *S. pneumoniae* and *H. influenzae* should be expanded to characterize the entire data set to develop an in depth profile for each isolate. The purpose of this would be to determine if serotype, ST or both mediate virulence. Additionally the virulence factor results could be parsed to determine if there is a core set of virulence factors required for a NVT to become widespread in the case of *S. pneumoniae*. An ideal use of this data-set would be as a carriage/disease comparison for the purpose of a pan-genome analysis, core genes present in both sets, could be investigated for the purpose of finding a new vaccine candidate that would reduce disease burden, not limited by serotype inclusion as well as the opportunity to reduce pneumococcal and NTHi carriage in general. Antibiotic resistance profiling should be continued but with the additional wet-lab testing of the isolates to determine if the antibiotic resistance genes detected in this study are expressed by the isolates to confirm resistance rates. The effect of a shared ecological environment of *S. pneumoniae* and *H.*

influenzae could be determined by identifying genetic changes that may or may not be occurring by analyzing strains that are co-carried together versus strains that are not co-carried. Finally, genetic relatedness of sequenced isolates could be further analysed through single nucleotide polymorphisms (SNP) typing.

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APPENDIX 1

Carriage study data (2006/07) SPN denotes *S. pneumoniae*

Study ID	***SPN***	**Study ID**	***SPN***	**Study ID**	***SPN***
0001		0056		0111	
0002		0057		0112	
0003		0058	1	0113	1
0004		0059		0114	
0005		0060		0115	
0006		0061	1	0116	
0007		0062		0117	
0008	1	0063		0118	
0009		0064	1	0119	
0010		0065		0120	
0011	1	0066		0121	
0012		0067		0122	
0013		0068	1	0123	
0014	1	0069		0124	
0015	1	0070		0125	
0016		0071	1	0126	
0017		0072		0127	
0018		0073		0128	
0019		0074		0129	
0020		0075	1	0130	
0021	1	0076		0131	
0022		0077		0132	
0023	1	0078		0133	

0024		0079		0134	
0025		0080	1	0135	1
0026	1	0081	1	0136	1
0027		0082	1	0137	
0028		0083		0138	1
0029		0084		0139	
0030		0085		0140	1
0031		0086		0141	1
0032		0087		0142	
0033		0088		0143	
0034		0089		0144	
0035		0090		0145	1
0036		0091		0146	
0037		0092		0147	
0038	1	0093	1	0148	
0039		0094	1	0149	
0040		0095	1	0150	
0041	1	0096		0151	
0042		0097		0152	1
0043		0098		0153	
0044		0099		0154	1
0045		0100		0155	1
0046		0101		0156	1
0047		0102		0157	
0048	1	0103	1	0158	
0049	1	0104		0159	1
0050	1	0105		0160	1
0051		0106		0161	
0052		0107		0162	
0053		0108		0163	1
0054		0109		0164	
0055		0110	1	0165	1
Study ID	***SPN***	**Study ID**	***SPN***	**Study ID**	***SPN***
0166		0221	1	0276	1
0167		0222		0277	
0168		0223	1	0278	
0169		0224		0279	1
0170		0225		0280	1
0171		0226		0281	1
0172	1	0227		0282	
0173	1	0228		0283	
0174	1	0229		0284	1
0175	1	0230		0285	1
0176	1	0231		0286	
0177		0232	1	0287	
0178		0233	1	0288	1
0179		0234		0289	1
0180		0235		0290	1
0181		0236		0291	1
0182		0237	1	0292	
0183		0238		0293	
0184		0239		0294	
0185		0240		0295	
0186		0241		0296	

0187		0242	1	0297	1
0188		0243		0298	
0189		0244		0299	
0190		0245	1	0300	
0191		0246		0301	1
0192		0247		0302	1
0193		0248		0303	1
0194	1	0249		0304	1
0195		0250		0305	1
0196	1	0251	1	0306	
0197		0252	1	0307	1
0198		0253		0308	1
0199		0254	1	0309	
0200		0255		0310	1
0201		0256	1	0311	1
0202		0257	1	0312	
0203		0258		0313	
0204		0259		0314	1
0205	1	0260		0315	1
0206		0261		0316	1
0207	1	0262	1	0317	1
0208		0263	1	0318	1
0209		0264		0319	1
0210		0265		0320	1
0211		0266	1	0321	1
0212		0267	1	0322	1
0213		0268	1	0323	
0214	1	0269		0324	1
0215	1	0270			
0216		0271			
0217		0272	1		
0218	1	0273	1		
0219		0274	1		
0220	1	0275			

Carriage study (2007/08) SPN denotes *S. pneumoniae*

Study ID	***SPN***	**Study ID**	***SPN***	**Study ID**	***SPN***	**Study ID**	***SPN***
2001	1	2056		2111		2166	1
2002	1	2057		2112		2167	
2003	1	2058	1	2113		2168	1
2004		2059		2114		2169	
2005		2060	1	2115		2170	
2006	1	2061		2116		2171	
2007		2062		2117		2172	
2008		2063	1	2118		2173	1
2009		2064		2119		2174	
2010		2065		2120		2175	
2011	1	2066		2121		2176	
2012		2067	1	2122	1	2177	
2013		2068		2123		2178	
2014	1	2069		2124		2179	
2015		2070	1	2125		2180	1

2016		2071		2126		2181	
2017		2072		2127		2182	
2018	1	2073	1	2128		2183	
2019		2074	1	2129		2184	
2020		2075		2130	1	2185	
2021	1	2076	1	2131	1	2186	1
2022		2077		2132		2187	
2023		2078		2133		2188	
2024	1	2079		2134	1	2189	
2025		2080		2135		2190	
2026		2081		2136		2191	1
2027	1	2082	1	2137		2192	
2028		2083		2138		2193	
2029	1	2084		2139		2194	
2030	1	2085		2140		2195	
2031		2086		2141		2196	1
2032	1	2087		2142	1	2197	
2033		2088		2143		2198	
2034	1	2089		2144		2199	1
2035		2090	1	2145		2200	
2036		2091		2146	1	2201	
2037	1	2092	1	2147		2202	
2038	1	2093		2148		2203	
2039		2094		2149	1	2204	
2040		2095		2150		2205	1
2041	1	2096		2151		2206	
2042		2097		2152		2207	
2043		2098		2153	1	2208	
2044		2099		2154		2209	
2045		2100		2155		2210	1
2046		2101		2156		2211	
2047		2102		2157		2212	
2048		2103		2158		2213	
2049		2104		2159		2214	
2050	1	2105	1	2160		2215	1
2051	1	2106		2161		2216	1
2052	1	2107		2162		2217	1
2053		2108		2163		2218	
2054	1	2109		2164		2219	
2055	1	2110		2165		2220	

Study ID	***SPN***	**Study ID**	***SPN***	**Study ID**	***SPN***
2221		2276		2332	
2222		2277		2333	1
2223		2278		2334	
2224		2279	1	2335	
2225	1	2280	1	2336	1
2226		2281		2337	
2227	1	2282		2338	1
2228		2283		2339	
2229		2284		2340	1
2230		2285		2341	
2231		2286		2342	
2232		2287	1	2343	1
2233	1	2288	1	2344	

2234		2289	1	2345	
2235	1	2290		2346	1
2236	1	2291		2347	
2237	1	2292		2348	
2238		2293		2349	
2239		2294		2350	
2240	1	2295		2351	1
2241		2296		2352	
2242		2297	1	2353	
2243	1	2298	1	2354	1
2244	1	2299		2355	
2245		2300	1	2356	
2246		2301		2357	
2247	1	2302		2358	
2248		2303		2359	1
2249		2304		2360	
2250	1	2305	1	2361	
2251		2306	1	2362	
2252		2307	1	2363	
2253		2308	1	2364	
2254	1	2309		2365	
2255	1	2310		2366	
2256		2311	1	2367	
2257		2312		2368	
2258	1	2313		2369	
2259		2314		2370	1
2260		2315	1	2371	1
2261	1	2316	1	2372	
2262		2317		2373	1
2263		2318			
2264		2319	1		
2265		2320			
2266		2321			
2267		2322			
2268		2323	1		
2269		2324	1		
2270		2325	1		
2271	1	2326			
2272		2327			
2273		2328			
2274		2329	1		
2275	1	2330			
		2331			

Carriage study 2008/09

Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**
3001	1					
3002						
3003	1					
3004				1		
3005				1		
3006	1			1		

3007						
3008						
3009	1					
3010						
3011						
3012						
3013				1		
3014						
3015						
3016	1					
3017	1				1	
3018		1				
3019						
3020	1					
3021	1					
3022	1					
3023						
3024	1					
3025						
3026	1	1				
3027						
3028		1				
3029	1					
3030						
3031	1					
3032	1					
3033	1					
3034		1				
3035						
3036					1	
3037						
3038	1			1		
3039						
3040		1				
3041						
3042	1	1				
3043						
3044						
3045		1				
3046		1		1		
3047		1				
3048	1	1				
3049						
3050	1					
3051						
3052						
3053						
3054						
3055	1					

Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**
3056						
3057						
3058						

3059	1					
3060	1					
3061		1				
3062						
3063	1	1			1	
3064	1					
3065	1					
3066	1					
3067		1			1	
3068	1	1				
3069						
3070						
3071						
3072						
3073						
3074	1	1				
3075						
3076						
3077	1	1				
3078						
3079	1					
3080						
3081	1					
3082						
3083						
3084						
3085						
3086						
3087						
3088	1					
3089						
3090						
3091						
3092				1		
3093	1					
3094						
3095						
3096						
3097	1					
3098						
3099	1					
3100						
3101						
3102						
3103						
3104						
3105						
3106						
3107						
3108	1					
3109	1			1		
3110		1				
3111						
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**

3112						
3113						
3114						
3115	1					
3116						
3117				1		
3118						
3119		1				
3120						
3121						
3122	1					
3123	1	1				
3124						
3125						
3126						
3127						
3128						
3129						
3130						
3131						
3132						
3133						
3134						
3135						
3136						
3137				1		
3138	1					
3139						
3140	1					
3141						
3142				1		
3143	1					
3144						
3145						
3146						
3147						
3148						
3149		1				
3150						
3151						
3152						
3153	1					
3154	1	1				
3155						
3156	1					
3157						
3158						
3159	1	1				
3160	1	1				
3161	1					
3162	1	1				
3163						
3164	1					
3165	1					
3166	1					

Study ID	*S. pneumoniae*	*H. influenzae*	*N. meningitidis*	AHS	MSSA	MRSA
3167						
3168				1		
3169						
3170						
3171	1			1		
3172						
3173						
3174						
3175						
3176	1					
3177						
3178						
3179		1				
3180	1					
3181	1					
3182	1					
3183						
3184						
3185						
3186		1				
3187						
3188					1	
3189						
3190	1					
3191						
3192	1					
3193						
3194						
3195		1				
3196		1				
3197		1				
3198						
3199						
3200	1	1				
3201					1	
3202						
3203		1				
3204					1	
3205	1	1				
3206				1	1	
3207		1				
3208						
3209						
3210	1					
3211		1				
3212						
3213						
3214		1				
3215					1	
3216						
3217					1	
3218		1				
3219						

3220	1					
3221						
3222						
3223						
3224		1				
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**
3225	1					
3226	1					
3227	1	1		1		
3228						
3229						
3230		1				
3231						
3232						
3233	1					
3234	1					
3235	1					
3236						
3237		1				
3238						
3239						
3240	1					
3241				1		
3242						
3243						
3244						
3245	1					
3246						
3247	1	1				
3248	1					
3249	1					
3250	1					
3251	1					
3252	1					
3253		1				
3254		1				
3255	1	1				
3256	1					
3257	1	1				
3258	1	1				
3259	1	1				
3260		1				
3261						
3262						
3263						
3264	1	1				
3265						
3266						
3267	1					
3268				1		
3269						
3270		1				
3271	1	1				
3272						

3273						
3274						
3275						
3276						
3277	1	1		1		
3278						
3279						
3280						
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**
3281						
3282						
3283	1					
3284	1					
3285		1				
3286	1					
3287	1					
3288		1				
3289						
3290						
3291		1				
3292						
3293	1	1				
3294		1				
3295						
3296						
3297						
3298	1	1				
3299	1					
3300	1			1		
3301	1					
3302						
3303						
3304						
3305						
3306						
3307						
3308						
3309		1				
3310		1				
3311						
3312						
3313					1	
3314	1					
3315	1					
3316						
3317					1	
3318						
3319						
3320						
3321	1					
3322						
3323						
3324	1					
3325	1					

3326	1					
3327						
3328						

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Study ID	*S. pneumoniae*	*H. influenzae*	*N. meningitidis*	AHS	MSSA	MRSA	Moraxella
4001							1
4002	1	1					
4003							1
4004							
4005							
4006							
4007							
4008	1				1		
4009				1	1		
4010					1		
4011	1						1
4012	1						1
4013		1					
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
4014							
4015	1				1		1
4016		1					
4017		1		1			1
4018				1			
4019				1			1
4020	1						
4021					1		
4022							
4023							1
4024	1						
4025							1
4026					1		
4027	1						
4028	1						1
4029	1						
4030							
4031							
4032	1			1			
4033							
4034				1			
4035							1
4036	1						1
4037		1					1
4038							
4039	1	1					1
4040							
4041					1		
4042							1
4043					1		
4044	1	1					1

4045					1		
4046							
4047							
4048					1		
4049	1						
4050							
4051				1			
4052							
4053				1			1
4054	1						1
4055							
4056							
4057							
4058							
4059					1		
4060							
4061							
4062							
4063							
4064							
4065							
4066					1		
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
4067					1		
4068							
4069							
4070							
4071	1						
4072							
4073							
4074	1						
4075							1
4076	1	1					
4077	1	1					
4078				1			1
4079							
4080					1		1
4081	1	1					
4082	1						
4083	1						1
4084	1	1					1
4085							
4086							1
4087	1						1
4088				1			
4089							
4090							1
4091	1	1					
4092							
4093				1			1
4094							
4095				1			1
4096	1						
4097							1

4098							1
4099	1						1
4100	1						
4101				1	1		
4102				1			
4103							1
4104	1			1			1
4105							
4106	1						1
4107							
4108				1			
4109	1						1
4110		1					1
4111							
4112							
4113							
4114							
4115					1		
4116		1					1
4117							
4118							
4119							
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
4120							
4121				1			
4122							
4123							
4124				1			1
4125				1			
4126							
4127							
4128							1
4129							1
4130							1
4131	1	1		1			
4132				1			
4133	1						1
4134	1						1
4135							
4136							1
4137							
4138	1	1					
4139	1						
4140		1		1			
4141				1			
4142							1
4143				1			
4144				1			
4145							
4146				1			
4147				1			
4148							1
4149							
4150	1						

4151	1	1					
4152		1					
4153							
4154		1					1
4155	1						
4156							
4157	1	1					
4158							
4159							
4160							1
4161							
4162							
4163		1					
4164	1						
4165	1	1		1			
4166	1						
4167	1	1					
4168	1						1
4169	1						
4170							
4171		1					
4172							
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
4173							
4174							
4175							
4176							1
4177							1
4178		1					
4179							
4180	1						
4181							
4182							
4183							
4184	1						1
4185							
4186				1			
4187	1	1			1		
4188							
4189							
4190		1					
4191	1						1
4192		1			1		
4193		1					
4194							
4195		1					
4196				1			
4197							
4198							
4199					1		
4200							
4201	1						
4202							
4203							

4204							
4205							
4206	1						
4207							
4208	1			1	1		1
4209							
4210							1
4211							
4212							
4213		1					1
4214	1						1
4215							
4216							
4217							
4218					1		
4219							1
4220							1
4221							
4222					1		
4223	1						1
4224					1		
4225							
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
4226	1	1					1
4227	1	1					1
4228							
4229							1
4230					1		1
4231							
4232							
4233							1
4234					1		
4235							
4236							1
4237	1						1
4238					1		
4239					1		
4240							
4241					1		
4242	1						
4244					1		
4245	1						
4246	1	1					
4247							
4248							
4249							
4250		1					
4251							
4252	1						
4253		1					
4254					1		
4255		1					
4256							
4257	1						

4258							1
4259					1		
4260				1			
4261							
4262				1			
4263		1					1
4264					1		
4265	1						
4266							
4267		1		1			
4268							1
4269	1						1
4270	1						
4272				1			
4273	1	1					
4274							
4275	1	1					
4276							1
4277							1
4278					1		
4279	1	1					
4280							
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
4281							
4282							1
4283				1			
4284							1
4285	1				1		
4286					1		
4287	1						
4288							
4289	1						
4290							
4291		1					
4292	1						1
4293				1			
4294	1						
4295					1		
4296				1			
4297	1	1					
4298		1					1
4299					1		1
4300	1						1
4301		1					1
4302							1
4303						1	1
4304							
4305		1					
4306		1					
4307							1
4308				1			1
4309							1
4310	1				1		1
4311	1						1

Study ID	*S. pneumoniae*	*H. influenzae*	*N. meningitidis*	AHS	MSSA	MRSA	Moraxella
4312							
4313							
4314	1	1					1
4315		1		1			1
4316							
4317							
4318	1						
4319							1
4320	1						
4321							
4322	1				1		1
4323					1		
4324							
4325				1			
4326	1				1		
4327				1			
4328							
4329							
4330	1						
4331		1					
4332							1
4333		1					
4334							
4335		1					1
4336							
4337	1	1					1
4338	1						1
4339	1			1			1
4340				1			
4341							1
4342	1						1
4343				1			
4344	1			1			1
4345		1					
4346	1	1					1
4347				1			
4348				1			1
4349							1
4350	1	1					1
4351				1			1
4352		1					
4353		1					
4354							
4355		1					
4356	1						1
4357							
4358							
4359							
4360							1
4361	1						
4362	1						
4363							
4364	1						

4365							
4366	1						
4367		1					
4368		1					
4369	1						
4370	1						1
4371	1						1
4372	1						
4373	1						
4374							
4375	1						1
4376				1			1
4377							1
4378		1					
4379		1		1			
4380	1	1					
4381							1
4382	1						
4383							
4384	1						
4385	1	1					
4386	1						
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
4387		1					
4388	1						
4389				1			1
4390				1			
4391							
4392							
4393	1	1					
4394				1			
4395				1			
4396		1					
4397							
4398							1
4399	1						
4400							1
4401							1

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Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitiids***	**AHS**	**MSSA**	**MRSA**	***M. catarrhalis***
5001				1			1
5002				1			1
5003	1						
5004							
5005							1
5006	1				1		
5007	1						1
5008							
5009				1			1

5010							
5011		1		1			
5012	1						
5013	1			1			1
5014	1						
5015	1	1			1		
5016	1						
5017							1
5018							
5019				1			
5020	1	1					
5021	1						1
5022	1						
5023	1	1					
5024							
5025							1
5026	1						
5027	1	1					
5028	1						
5029							
5030	1						
5031							
5032		1					
5033	1						1
5034		1					1
5035							
5036							
5037							
5038	1						
5039		1					1
5040	1						
5041							
5042		1		1			
5043	1						1
5044							
5045				1			1
5046							
5047	1			1			
5049				1			1
5050	1						1
5051	1						1
5052							1
5053	1						
5054							
5055							1
5056							
5057	1						
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
5058							1
5059	1						1
5060							
5061	1				1		
5062	1						
5063							1

5064					1		
5065	1						1
5066	1						
5067							
5068	1						
5069							
5070							1
5071		1					
5072	1						
5073							
5074	1						
5075							
5076				1			
5077				1			
5078		1					
5079	1	1					
5080	1	1					
5081							
5082							
5083	1						
5084		1					
5085							
5086							1
5087							
5088							
5089	1						
5090							
5091							
5092							
5093				1			
5094							
5095							
5096				1			
5097							1
5098	1						1
5099							
5100				1			1
5101							1
5102		1		1			
5103				1			
5104							
5105							1
5106							1
5107							1
5108	1						1
5109	1						1
5110	1						
5111		1					1
5112		1					1
5113	1	1					
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
5114	1						
5115		1		1			1
5116							

5117							
5118		1					
5119	1			1			1
5120	1			1			1
5121	1						
5122	1	1					1
5123							1
5124	1						
5125							1
5126		1					1
5127							
5128							
5129	1						
5130		1					
5131							
5132				1			
5133	1	1					
5134		1					1
5135	1	1			1		
5136	1						1
5137							
5138							
5139		1		1			
5140							
5141							
5142	1	1					
5143							
5144	1			1	1		
5145							
5146							
5147							
5148				1			
5149	1						
5150							
5151		1					
5152	1	1					
5153				1			
5154				1			
5155		1					
5156	1	1					
5157	1						
5158							
5159	1	1					
5160							
5161				1			
5162		1					
5163	1						
5164							1
5165	1	1					1
5166	1						1
5167				1	1		
5168		1		1			
5169							
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**

5170	1				1		
5171	1			1	1		
5172							1
5173	1						1
5174							1
5175		1					
5176							1
5177							1
5178	1						1
5179							1
5180	1			1			
5181	1	1					
5182				1			1
5183				1			
5184		1					1
5185		1					
5186	1						
5187				1			1
5188							
5189	1						
5190							
5191	1						
5192		1					
5193	1						
5194		1					1
5195							
5196							1
5197		1					1
5198							1
5199		1		1			
5200							
5201							
5202	1						
5203	1						1
5204							
5205	1						
5206		1					1
5207							
5208							1
5209	1	1					1
5210				1			1
5211							
5212							1
5213				1			
5214							
5215					1		
5216		1					
5217	1						
5218	1						
5219		1					
5220	1	1					
5221							
5222	1						1
5223				1			1
5224		1					

5225	1						
5226	1	1					
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
5227	1	1					1
5228							
5229		1					
5230		1					
5231				1			
5232	1	1					
5233	1	1					1
5234	1	1					1
5235	1						
5236							
5237		1					
5238		1					
5239		1					
5240							
5241							
5242							
5243	1						
5244							1
5245							
5246							
5247	1				1		
5248	1						
5249	1						
5250					1	1	
5251	1				1		
5252							
5253							
5254		1					1
5255				1	1		
5256				1			
5257							
5258							1
5259	1	1					
5260							1
5261				1			1
5262	1	1					1
5263	1						1
5264							
5265	1	1					
5266				1			1
5267	1						
5268							1
5269	1						1
5270	1						1
5271	1						1
5272				1			1
5273				1			
5274	1						
5275	1						1
5276	1						1
5277					1		1

5278							
5279		1		1			1
5280		1		1			
5281					1		1
5282							
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
5283							
5284	1	1					1
5285	1						1
5286							
5287	1	1					1
5288	1	1			1		1

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Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitiidis***	**AHS**	**MSSA**	**MRSA**	***M. catarrhalis***
6001							
6002							
6003	1						
6004							1
6005							
6006							
6007							
6008							1
6009					1		1
6010	1						
6011							
6012	1						
6013							
6014							1
6015	1						1
6016							1
6017							1
6018					1		
6019				1			
6020	1						1
6021	1	1					
6022	1						
6023	1						1
6024				1			
6025	1						1
6026	1						1
6027	1						
6028							
6029							1

6030							1
6031		1					1
6032	1	1					
6033							1
6034							
6035	1						
6036	1						
6037	1						
6038							
6039				1			1
6040							1
6041							
6042	1	1					1
6043							1
6044	1						1
6045							1
6046				1			
6047							
6048							
6049							1
6050	1						
6051	1						
6052	1						1
6053							1
6054	1	1					1
6055	1						
6056							
Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
6057							
6058	1						
6059				1			
6060							
6061	1						
6062	1						1
6063		1					
6064							
6065							
6066							
6067	1						1
6068				1			1
6069					1		
6070							
6071							
6072							
6073							1
6074					1		1
6075							
6076							1
6077							1
6078	1						1
6079	1						1
6080							
6081							
6082			1				1

6083		1					
6084				1			
6085					1		
6086							
6087			1				1
6088							
6089							
6090	1						
6091		1					
6092							1
6093					1		
6094	1						1
6095	1						1
6096							
6097							
6098							
6099							
6100	1	1					
6101	1	1			1		1
6102							
6103							
6104							
6105							
6106	1	1					
6107	1	1					
6108							
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Study ID	***S. pneumoniae***	***H. influenzae***	***N. meningitidis***	**AHS**	**MSSA**	**MRSA**	**Moraxella**
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APPENDIX 2

APPENDIX 2. 1 PNEUMOCOCCAL VIRULENCE FACTOR DETAILS

Gene details derived from http://www.mgc.ac.cn/VFs/download.htm

Name	Characteristics	Function
Autolysin	Surface protein: choline-binding proteins anchored to the cell surface by a non-covalent interaction of a repeat region at the C-terminal with the phosphorylcholine of the cell wall; Attachment of the enzyme to the choline of the S. pneumoniae cell wall teichoic acid is essential for the lytic activity of the enzyme; two other cell wall hydrolases, LytB and LytC have recently been described, but their roles in virulence have not been assessed. LytB plays a role in pneumococcal daughter cell separation. LytC has a lysozyme-like activity	N-acetylmuramoyl-L-alanine amidase: degrading the peptidoglycan backbone of the bacteria, leading to the release of the component of cell wall shown to be highly inflammatory and the release of cytoplasmic bacterial proteins including bacterial virulence factors such as pneumolysin
Capsule	Ninety different capsule types have been identified. Each has a structurally distinct capsule, composed of repeating oligosaccharide units joined by glycosidic linkages	Resistant to complement deposition and masks cell wall-associated complement from being recognized by the complement receptors on phagocytes
CbpA	Also known as PspC, SpsA, Hic (factor H-binding inhibitor of complement); surface protein: choline-binding protein	Mediate adherence to sialic acid and lacto-N-neotetraose ligands present on cytokine-activated epithelial cells; specifically binds the secretory component of human secretory immunoglobulin A, human factor H, and complement component C3; involved in immune-cell recruitment and chemotaxis by stimulating the production of IL-8 from pulmonary epithelial cells; interacts with the human polymeric immunoglobulin receptor (pIgR), pIgR domain D3 and D4 together are necessary and sufficient for the ligand-binding activity. The poly-immunglobulin receptor (pIgR) of mucosal epthelial cells mediates the transport of pIgA across polarized epithelial cells, resulting in release of secretory component (SC), either free or bound covalently to IgA. A hexapeptide motif of CbpA has been shown to interact in a human specific manner with the SC/pIgR.
CBPs	Surface protein: choline-binding	CbpD, CbpE, CbpG, LytB and LytC play a role in adhesion and colonization of the nasopharynx

Hyaluronate lyase	Surface protein: LPXTG-anchored proteins	Facilitates tissue invasion by breaking down the extracellular matrix (ECM) components
IgA1 protease	Two more zinc metalloproteinase ZmpB and ZmpC may also contribute to virulence	Cleaves the Pro227-Thr228 peptide bond in the IgA1 hinge; may enable pathogens to subvert the antigen specificity of the humoral immune response to facilitate adhesive interactions and persistence on the mucosal surface
Neuraminidase	Surface protein: LPXTG-anchored; two forms of the pneumococcal neuraminidase enzymes, NanA and NanB. The activity of NanB is approximately 100 times lower than that of NanA. NanA but not NanB contains a LPXTG motif in C-terminal, the sequence similarity between the two enzymes is only 20%; maximum activity: NanA at ~pH5, NanB at ~pH7	Cause significant damage to host cell glycans, change the glycosylation patterns of the host and probably exposes more of the host cell surface, which may reveal surface receptors for possible interaction with the bacteria, contributing to increased adhesion
Pneumolysin	Shares amino acid homology with similar hemolysins such as SLO, LLO, but pneumolysin is a cytoplasmic protein rather than a secreted protein	Multi-function enzyme:;<spacer>forming pore in the host cell membranes;<spacer>inflammatory and proapoptotic effects: stimulating the production of inflammatory mediators, such as TNF-<alpha>, IL-1<beta>, nitric oxide, IL-8, and prostaglandins and leukotrienes;<spacer>inhibiting nonspecific defences: inducing ciliary stasis;<spacer>evasion of immune system: antiphagocytosis and interferes with the complement pathway
PsaA	Surface protein: lipid-attached; AdcABC is another ABC transporter for Zn2+ uptake	Key function is the transport of Mn2+ and Zn2+ into the cytoplasm of the bacteria; psaA mutants show marked impact on the capacity to colonize and increased susceptibility to oxidative damage

PspA	Surface protein: choline binding protein	Inhibits complement activation and reduces the effectiveness of complement receptor-mediated clearance mechanisms by preventing the deposition of C3b on the cell surface; binds to lactoferrin, the iron starage glycoprotein predominantly located in mucosal secretions, may be involved in iron acquistion