Epidemiologic Characteristics of Colonizing *Streptococcus pneumoniae* in Vietnam and Implications for Population Vaccination

by

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For my husband, Charlie, with love and gratitude for his unwavering support and belief in me.
And for my daughter, Ella, for filling my life with love and laughter.

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Abstract

Streptococcus pneumoniae is the leading cause of bacterial infections worldwide and causes a range of illness from otitis media to pneumonia and meningitis. Use of the heptavalent pneumococcal conjugate vaccine (PCV7) in the United States led to a reduction in invasive pneumococcal disease caused by vaccine serotypes (VTs), reduced nasopharyngeal (NP) carriage of VTs, and reduced antibiotic-resistant pneumococcal disease. However, an increase in colonization and disease due to non-vaccine serotypes has been observed and may threaten the long-term utility of this vaccine. Other countries now want to introduce a PCV. Vietnam, in particular, would like to introduce a PCV, but the epidemiologic data upon which to base this decision are lacking.

In order to provide Vietnamese health policymakers with the data upon which to base a decision about a pneumococcal vaccine and to more broadly advance the understanding of the epidemiology of *S. pneumoniae*, we conducted a cross-sectional study of NP carriage among 519 healthy individuals of all ages who lived in 115 households in one hamlet of Nha Trang, Vietnam. NP carriage was common among children (40%), and colonization decreased with age to 2% among adults ≥ 50 years. Children ≤5 years were more likely to carry PCV serotypes, while older children and adults were more likely to carry non-PCV serotypes. The prevalence of multidrug resistance was high among PCV and non-PCV serotypes (83%). We also identified an unusually high proportion of nontypeable (NT) pneumococci colonizing children and

adults in Vietnam (30% of isolates). These NTs were more likely to be resistant compared to encapsulated pneumococci. Furthermore, we demonstrated that a multiplex PCR assay for capsular typing of invasive isolates can also be used with carriage isolates. Finally, we identified age as the most important predictor of individual- and household-level pneumococcal colonization.

Children in Vietnam may greatly benefit from use of a PCV, although a vaccine may not reduce antibiotic resistance in Vietnam in the long-term because of the high prevalence of resistance among non-PCV serotypes and the low probability of decreases in inappropriate antibiotic usage in Vietnam.

Chapter 1

Introduction

Streptococcus pneumoniae is the leading cause of bacterial infections worldwide; the bacterium can cause a range of illness from otitis media and sinusitis to invasive disease, such as meningitis and sepsis (1). Each year, approximately half of the 2.6 million deaths due to acute respiratory infections in children under five are due to pneumococcal pneumonia (10, 11). Invasive pneumococcal disease (IPD, pneumonia, meningitis, and sepsis) in children is particularly associated with mortality and is a major cause of hospitalizations and long-term neurologic sequelae (14).

S. pneumoniae is a naturally transformable, encapsulated diplococcus that is considered a normal constituent of the human nasopharyngeal flora. Healthy children commonly carry at least one serotype of *S. pneumoniae* in their nasopharynges without developing disease (11). Asymptomatic carriage of *S. pneumoniae* is central to the organism's transmission, and colonization is frequently followed by horizontal spread of the pneumococci through an individual's immediate environment and the surrounding community (2). Pneumococcal disease will not occur without preceding nasopharyngeal colonization with the homologous strain, although the precise mechanism(s) by which colonizing *S. pneumoniae* travels to other anatomical sites and becomes pathogenic is poorly understood (2, 11). Based on differences in the composition of the polysaccharide capsule, at least 91 serotypes of *S. pneumoniae* have been identified (13). The

polysaccharide capsule is considered the main virulence factor, and antibodies directed against it facilitate opsonophagocytosis.

In 2000, a heptavalent pneumococcal conjugate vaccine (PCV) was licensed in the United States (Prevnar®) for routine use in children 2-23 months of age and among children up to five years who are at high risk for pneumococcal disease. PCV7 is a formulation of the seven most prevalent pneumococcal capsular types in the United States (4, 6B, 9V, 14, 18C, 19F, and 23F) conjugated to a nontoxic variant of the diphtheria toxin. The most prevalent penicillin-resistant pneumococcal strains are also of the serotypes that are included in the conjugate vaccine. Use of PCV7 in the United States led to a dramatic reduction in the incidence of IPD caused by vaccine-targeted serotypes (18). Unlike the older 23-valent polysaccharide pneumococcal vaccine, the conjugate vaccine reduces carriage by preventing new nasopharyngeal acquisition of vaccine serotypes and, occasionally, of those serotypes that are cross-reactive to those in the vaccine. Consequently, use of PCV7 in young children created a herd effect by reducing transmission of vaccine serotypes and led to a population-level decrease in IPD. PCV7 also reduced the incidence of antibiotic-resistant pneumococcal disease in vaccinees and some non-vaccinated children and adults, because the serotypes included in PCV7 are frequently antibiotic resistant (7).

Concomitant with reductions in carriage and disease due to vaccine-type *S. pneumoniae*, an increase in colonization and disease due to non-vaccine serotypes (i.e., serotype replacement) has been observed in all studies that examined colonization and in most disease studies (3-6, 9, 12, 17, 19). Although the increase in non-vaccine disease

still remains small compared to the benefits gained by PCV7, the serotype replacement phenomenon may threaten the long-term utility of this vaccine.

Due to the overall success of PCV7 in the United States, other countries are now eager to introduce a pneumococcal conjugate vaccine (PCV) into their pediatric populations. Additional PCVs with expanded serotype coverage are available or will be brought to market shortly. Vietnam, in particular, is eligible for financial support for a PCV and has expressed interest in introducing a PCV in young children. However, there is limited information on the disease burden and distribution of pneumococcal serotypes causing disease in Vietnam. Antibiotic resistance is also a particular problem in Vietnam, which has one of the highest rates of penicillin and macrolide resistant pneumococci of all Asian countries (8, 15, 16). It is widely anticipated that use of a PCV could be a novel way to dramatically decrease antibiotic resistance, at least temporarily. The success of a PCV in Vietnam will depend on the percent of individuals of all ages who are colonized with pneumococci, the pre-vaccine distribution of PCV and non-PCV serotypes carried by all age groups, the frequency of antibiotic resistance among PCV and non-PCV serotypes, and social mixing patterns.

The overarching goal of this dissertation was to describe the epidemiology of colonizing *Streptococcus pneumoniae* among individuals in Vietnam, with the objective of providing the necessary data upon which health policymakers in this country could base a rational decision about an appropriate PCV for infants and children. By including individuals of all ages in our studies, we also more broadly aimed to shift the focus of infectious disease epidemiology from individual-level risk assessment to a systems approach that centers on the transmission of infectious agents through a population and

how the role of different age groups and contact networks will shape disease patterns. Specifically, the aims of this dissertation are as follows:

1. Describe the prevalence of pneumococcal colonization among healthy individuals of all ages living in households in Nha Trang, Vietnam.
2. Determine the serotype distribution of pneumococci carried by individuals of all ages living in households in Nha Trang, Vietnam.
3. Establish the prevalence of antibiotic resistance among colonizing pneumococci carried by individuals of all ages living in households in Nha Trang, Vietnam.
4. Employ the colonization, serotyping, and antibiotic resistance data to recommend a PCV for use in infants and young children in Vietnam.
5. Describe the prevalence of nontypeable pneumococci carried by individuals of all ages living in households in Nha Trang, Vietnam.
6. Determine if a recently developed multiplex PCR assay for capsular typing of invasive disease isolates can be applied to carriage isolates.
7. Establish which of three formulations of the multiplex PCR assay will most accurately type pneumococci from Vietnam.
8. Identify individual- and household-level characteristics associated with pneumococcal colonization in Vietnam.

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Chapter 2

Background and Significance

Global burden of invasive pneumococcal disease (IPD)

Streptococcus pneumoniae is the leading cause of bacterial infections worldwide, ranging from common infections, such as otitis media, to invasive disease (11). Each year, approximately half of the 2.6 million deaths due to acute respiratory infections (ARIs) in children under five are due to pneumococcal pneumonia (84, 90). Invasive pneumococcal disease (IPD) (pneumonia, meningitis, and sepsis) in children is particularly associated with mortality and is a major cause of hospitalizations and long-term neurologic sequelae (111). Previous studies conducted in developed countries yielded IPD incidence rates ranging from 20 to 80 cases/100,000/year in children less than five years old. For example, Finland reported an under-five IPD incidence of 45/100,000/year (41). Higher IPD rates in children under five years were found in New Zealand and the United States, who reported incidences of 60/100,000/year and 80/100,000/year, respectively. In particular, the highest rates of IPD have been found among children less than two years of age (50 to 150/100,000/year) (13, 63, 68, 110). In Spain, the incidence of IPD in children under two years was reported to be 59.6/100,000/year (36).

Parallel estimates of IPD incidence are difficult to obtain for less developed nations, owing to poor public health infrastructure, a lack of systematic disease surveillance, and minimal microbiologic laboratory capacity. Specifically in Asia, the burden of IPD remains largely undocumented. A limited number of studies have recently suggested that *S. pneumoniae* is a common cause of bacterial meningitis in children (61). Investigations from China, Viet Nam, the Philippines, and Thailand demonstrated that incidence rates for pneumococcal meningitis are second only to that of *Haemophilus influenzae* type b (Hib) in children less than five years old (72, 76, 111, 134). As numerous Asian countries already have or are considering incorporating the Hib vaccine into their immunization schedules, *S. pneumoniae* will become the leading cause of community-acquired invasive disease among children in this region. The advent of the pneumococcal conjugate vaccines holds promise for reducing the pneumococcal disease burden, especially for children less than two years old. However, no studies in Asia have systematically characterized the incidence of IPD in children, and information upon which Asian health policymakers can make decisions regarding pneumococcal vaccination is lacking.

Epidemiology of *Streptococcus pneumoniae*

S. pneumoniae is a naturally transformable, encapsulated diplococcus that is considered a normal constituent of the human nasopharyngeal flora. Based on differences in the composition of the polysaccharide capsule, 91 serotypes of *S. pneumoniae* have been identified. The polysaccharide capsule is considered the main virulence factor, and antibodies directed against it facilitate opsonophagocytosis.

Transmission of *S. pneumoniae* occurs via direct contact with respiratory droplets from persons with active pneumococcal disease or, more commonly, from persons who asymptomatically carry the organism in the nasopharynx (86). Following exposure, *S. pneumoniae* may establish itself in the nasopharynx of the new host; most often, transient asymptomatic colonization results, and the microorganism is carried for weeks to months. Under certain conditions, however, the newly acquired organism may evade the host immune defenses and cause disease. Data suggests the risk of disease is greatest shortly after acquisition of a new serotype in the nasopharynx, although illness may also occur after months of colonization (19). Humans are the only known reservoir for *S. pneumoniae*; the incubation period is not well-defined and may be as short as 1-3 days (1). The most common clinical manifestations of *S. pneumoniae* infection are considered due to either mucosal or invasive infections. Mucosal infections most often involve migration from the upper respiratory tract (e.g., acute otitis media and acute bacterial sinusitis). Invasive pneumococcal disease occurs when the microorganism is isolated from a normally sterile site, such as the lower respiratory tract (pneumonia), the bloodstream (bacteremia or sepsis), or the central nervous system (meningitis) (19).

Risk factors for IPD

Host factors that have been consistently associated with an increased risk of IPD across geographic locales include: young age, day-care attendance, lower socioeconomic status, underlying immunodeficiency and other chronic medical conditions, preceding or coincident respiratory viral infection, and recent antibiotic usage. Previous studies have shown the incidence of IPD is greatest among children less than two years old. In

particular, the highest rates of disease occur among children 6-11 months of age (19); neonatal infections are not uncommon in developing countries. The increased risk of IPD among children less than two years old is postulated to be due to an immature immunologic response to the polysaccharide capsule of *S. pneumoniae* and the high prevalence of colonization among this age group (19).

Day-care or school attendance or having a sibling that attends day-care has been strongly associated with IPD (44, 127). Day-care center (DCC) attendance has specifically been cited as a predisposing factor for IPD due to resistant pneumococci (89). Owing to the close interactions between children, it has been proposed that pneumococci are more efficiently transmitted from the nasopharynx of one child to another in DCCs. The higher rate of disease in DCCs results in increased antibiotic usage, selecting for the carriage of resistant pneumococci. Indicators of socioeconomic deprivation, such as low household income, minimal education, and crowding, have been identified as risk factors for IPD. Within-household crowding and exposure to children/siblings, in particular, have been utilized as markers of increased transmission of pneumococcal serotypes.

Individuals with functional or anatomic asplenia (e.g., sickle cell disease or splenectomy) are at a higher risk for pneumococcal infection due to reduced clearance of encapsulated bacteria from the bloodstream (89). The presence of other chronic medial conditions, such as HIV infection, chronic obstructive pulmonary disease, asthma, diabetes mellitus, cancer, and people on immunosuppressive therapies, individuals with congenital or traumatic cerebrospinal fluid fistulae, and children who have cochlear implants are associated with a higher risk of IPD (19, 128). These medical conditions are characterized by a state of immune compromise, anatomical abnormalities, and/or altered

physiology of the respiratory tract, which facilitate opportunistic infection with *S. pneumoniae* (55).

Cases of IPD are also more common during colder weather months, and IPD tends to follow the annual cyclic pattern of influenza and other viral respiratory infections (37, 46, 62). In particular, an association between IPD and influenza A, parainfluenza, and respiratory syncytial viruses has been observed. *S. pneumoniae* has multiple neuraminidases that cleave terminal sialic acid, potentially exposing cell-surface receptors and promoting adherence for pneumococci. Viruses with neuraminidase activity, such as influenza and parainfluenza, may act synergistically with the pneumococcus to promote adherence to the respiratory epithelium. Increased risk of pneumococcal infection during respiratory viral infection may also be due to inhibition of the normal clearance mechanisms of the respiratory tract, epithelial barrier damage, or serve as a marker of increased transmission due to indoor crowding during cooler weather (143).

Finally, previous antibiotic usage has been associated with an increased risk of IPD caused by a resistant strain of pneumococcus. Specifically, prior treatment with β-lactam antibiotics has been associated with IPD due to a drug-resistant strain. In a case-control study of children 2-59 months old, IPD due to a penicillin-resistant pneumococcal strain was independently associated with at least one course of antibiotics in the previous three months (71, 89). Prior antibiotic usage may alter an individual's nasopharyngeal flora and increase the likelihood of carrying resistant pneumococci that can cause IPD under opportunistic conditions.

Pathogenesis and Treatment

Although colonization with pneumococci is mostly asymptomatic, it can progress to respiratory or systemic disease. An important feature is that pneumococcal disease will not occur without preceding nasopharyngeal colonization with the homologous strain (15). Many of the characteristics or factors that increase the likelihood of pneumococcal colonization (e.g., young age, underlying immunodeficiency, crowding, and exposure to young children) are the same characteristics that increase individual risk for pneumococcal disease. An individual who is susceptible to colonization with a pneumococcus may become colonized upon exposure and can carry the strain for weeks-to-months. Invasive disease most commonly occurs following acquisition of a new serotype, typically after an incubation period of 1-3 days (25). However, the mechanisms by which colonizing pneumococci become pathogenic and the processes involved in the translocation of the pneumococcus from the nasopharynx to other anatomic sites are poorly understood (18, 56, 90).

Colonization by *S. pneumoniae* requires adherence to the epithelial lining of the respiratory tract (15). It has been proposed that attachment of pneumococci to the respiratory epithelium is mediated by a disaccharide receptor on fibronectin, which is present on all epithelial cells (6). Additionally, adherence of pneumococci to tracheal epithelial cells may be enhanced by prior influenza or parainfluenza virus infection, most likely mediated by viral neuraminidase (104). This enzyme cleaves sialic acid from glycosphingolipids, which are found in substantial amounts in human lung tissue (67). Thus, neuraminidase is thought to expose other structures that function as receptors to which pneumococci can adhere (90).

Three interchangeable variants of pneumococci have been described, based on colony morphology: opaque, semi-transparent, and transparent; these phenotypes appear to have different abilities to colonize the nasopharynx and cause different types of disease, but the biochemical basis for the phenotypic variation is unknown (144). The bacterium interacts with the glycoconjugate that serves as a receptor on eukaryotic cells in the nasopharynx (90). Phase variation appears to play a role in the adaptation of pneumococci to changes in receptors presented on activated host cells (27). The transparent phenotype appears to have an advantage in colonization of the nasopharynx, due to its ability to recognize the cognate eukaryotic ligand (G1cNAcβ1-3Gal) (26). More adhesive strains cause localized infections, and less adhesive strains cause invasive disease, such as bacteremia and meningitis (90). Translocation of the organism by either aspiration or penetration of the mucosa results in bacteremia or sepsis, or both, and seeding in different anatomic body parts may cause disease, such as meningitis and arthritis (90).

Once pneumococci attach to and invade host tissues, disease is primarily caused by replication within host tissues and the subsequent inflammatory response (125). Unlike many other pathogens (e.g., *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Escherichia coli*, and *Clostridium difficile*), pneumococci produce relative few exotoxins (133). The host responds to cell wall components, such as teichoic acids that are released after bacteriolysis and to pneumolysin that is released during bacterial growth and lysis (45, 125). Certain proteins or enzymes displayed on the surface of pneumococci (e.g., hyaluronate lyase, autolysin, neuraminidases, PsaA, and other choline-binding proteins) may contribute to the pathogenesis and disease symptoms.

It is generally thought that pneumonia results from the aspiration of pneumococci from the upper respiratory tract, although a blood-born route of dissemination from the upper respiratory tract is also possible (18). Pneumococci are presumably aerosolized from the nasopharynx to the alveolus, bypassing the ciliated epithelium, to which they cannot attach (137). Bronchopneumonia, which involves airways more than alveoli, is promoted by antecedent injury to the ciliated mucosa, providing conditions for pneumococcal attachment (136). This tropism of pneumococci for damaged epithelium was shown in autopsy materials from patients dying of pneumonia during the influenza pandemic of 1957-1958 (75). Damaged epithelium can also arise by smoking or infection with other respiratory pathogens (136). Exposure of the endobronchial submucosa allows pneumococci to attach to basement membrane components, such as fibronectin and collagen (136). Once localized to the airway, release of pneumolysin exacerbates the mucosal damage, since epithelial cells are particularly sensitive to toxin-induces cytolysis and inhibition of ciliary beat frequency (136).

Pneumococcal meningitis usually arises in the context of sustained bacteremia that permits bacterial localization to and transit across the blood-brain barrier (BBB) into the subarachnoid space (141). The central pathologic event in meningitis is breakdown of the BBB. Bacteria penetrate from the blood across the tight junctions of the cerebral vessels and multiply upon entry into the cerebrospinal fluid (CSF), a site sequestered from host defenses (141). Once in the CSF space, multiplication of bacteria results in release of cell wall components and toxins (pneumolysin and hydrogen peroxide). Bacterial surface fragments, including cell wall, incite direct host cell toxicity and the release of a broad range of cytokines that serve to increase inflammation, alter vascular

permeability, and induce influx of leukocytes (43, 139). Over a period of hours, gaps are opened between endothelial cells, pinocytotic activity increases, leukocytes are recruited to the CSF, and cytochemical parameters in CSF change (138). Injury to the cerebral microvasculature promotes brain edema that in turn leads to intracranial hypertension. In addition, oxygen and nitrogen intermediates released by the inflammatory process cause loss of autoregulation of cerebral vascular perfusion pressure with resultant ischemia (103). The precise events that lead to neuronal damage remain unclear, but both direct toxicity and injury from host-derived inflammation are involved (129).

While the exact mechanisms underlying attachment of pneumococci to mucosal surfaces, invasion of host tissues, and pathogenesis remain elusive, antibiotics are the foundation of treating pneumococcal disease. Pneumococcal disease is primarily treated with penicillin or a cephalosporin. In recent years, resistance to penicillin and other commonly used antibiotics have increased, making treatment difficult and resulting in longer hospitalizations and more expensive therapies. All isolates of *S. pneumoniae* were uniformly sensitive to penicillin until outbreaks of antibiotic-resistant pneumococci occurred in South Africa in the late 1970s (7, 54). Since then, pneumococci have developed resistance to several classes of antibiotics, including: β-lactams, macrolides and lincosamines, tetracyclines, trimethoprim-sulfamethoxazole, glycopeptides, and fluoroquinolones.

Most patients with acute otitis media and community-acquired pneumonia are treated empirically. When there is a positive culture for *S. pneumoniae*, the antibiotic susceptibility pattern guides therapy. Oral amoxicillin is the recommended first-line therapy for acute otitis media in children. For clinically-defined treatment failures, oral

amoxicillin-clavulanate, oral cefuroxime axetil, oral cefdinir, or a intramuscular injections of ceftriaxone are recommended (39). Penicillin or ampicillin is the drug of choice for pneumococcal pneumonia caused by isolates with penicillin MICs ≤2 μg/mL (20, 24, 33). Cefuroxime, cefotaxime, or ceftriaxone can be used to treat children with pneumococcal pneumonia caused by isolates that are susceptible to the particular cephalosporin (58). The increased incidence of strains intermediately or fully resistant to penicillin has raised questions about the predictable efficacy of penicillin or cephalosporins. Some clinicians have argued that increasing the dose to 24 million units of penicillin per day or using ceftriaxone or cefotaxime is sufficient for pneumonia caused by intermediately susceptible and low-level resistant strains (105). However, β-lactams should be avoided when the MIC is >2 μg/mL (95). Cefotaxime or ceftriaxone still are effective for treating pneumococcal pneumonia caused by isolates with MICs up to 2 μg/mL and possibly up to 4 μg/mL, but data on this estimate are lacking because there are few strains with MICs this high. Linezolid and clindamycin (if the isolate is susceptible) are additional options for treating infections caused by highly resistant isolates. Clones of the pneumococcal serotype 19A, in particular, may be multidrug resistant (penicillin, macrolides, clindamycin, and trimethoprim-sulfamethoxazole). In these cases, vancomycin or linezolid may be the only agents that have approval in children with antimicrobial activity against the isolates (85, 102).

Empiric treatment for suspected bacterial meningitis is initiated immediately after the results of the lumbar puncture (LP) have been obtained, or immediately after LP alone if suspicion of bacterial meningitis is high. Coverage for *S. pneumoniae* is included in the empiric antibiotic regimen for infants and children older than 1 month with

suspected bacterial meningitis, since *S. pneumoniae* the most common cause of bacterial meningitis this age group, even for children who have completed immunization with the pneumococcal conjugate vaccine (57). Because of the increasing prevalence of antibiotic-resistant strains of pneumococcus, the empiric regimen includes vancomycin until culture results are available and the isolated pathogen is confirmed to be susceptible to penicillin, cefotaxime, or ceftriaxone (3, 99, 102). No pneumococcal isolates resistant to vancomycin have been reported. However approximately 3% of isolates are tolerant to vancomycin, and vancomycin-tolerant pneumococcus has been associated with treatment failure in at least one patient (52, 82). Empiric therapy is usually altered when pneumococci are isolated from CSF culture and the antimicrobial susceptibility pattern is known. The therapeutic options for pneumococcal meningitis in children and adults include: penicillin, cefotaxime or ceftriaxone, vancomycin, and rifampin (which can be added to vancomycin if the pneumococcal isolate has a particularly high MIC for the cephalosporins, vancomycin appears to be failing, or the patient continues to have organisms present in repeat LPs) (99). Newer generation quinolones, such as moxifloxacin and gemifloxacin, with enhanced activity against pneumococci are effective in animal models of antibiotic-resistant pneumococcal meningitis and may prove to be alternative agents for treating this infection in humans (77). Fluoroquinolones currently are not recommended for use in children < 18 years because of effects on growing cartilage in experimental animals. However, for serious infections, such as meningitis, these drugs may be indicated if other agents cannot be used (4). Chloramphenicol also has been used for patients with an allergy to penicillin and cephalosporin. Unfortunately, many penicillin-resistant strains are somewhat resistant to chloramphenicol killing, and

treatment failures of meningitis caused by penicillin-resistant *S. pneumoniae* have occurred when chloramphenicol was used (66).

The emergence of antibiotic resistant pneumococci and a few internationally-spreading multidrug resistant clones has placed further emphasis on the prevention of pneumococcal disease through vaccination. In particular, the recently licensed heptavalent pneumococcal conjugate vaccine (PCV7) is widely anticipated to reduce the incidence of antibiotic resistant pneumococcal disease, because the serotypes included in the vaccine are frequently associated with resistance. The ability of vaccination with PCV7 among infants and young children to reduce antibiotic resistant pneumococcal infections long term (especially if there are little or no changes in antibiotic usage practices) remains to be seen. The long-term effect of any PCV on resistance will differ by geographic location and will be dependent on the pre-vaccine serotype distribution and antibiotic susceptibility pattern among pneumococci carried by all individuals in a population.

Acute otitis media

Acute otitis media (AOM) is the most common infection following pneumococcal colonization and accounts for substantial morbidity and healthcare costs, globally (112, 113). AOM is the most common reason for children to be brought to medical care in the United States, representing approximately 25% of pediatric visits and more than 20 million doctor visits per year (38). A longitudinal study of 698 children in Boston, Massachusetts, demonstrated that 83% of children experienced one or more episodes of AOM by three years of age (130). In the United States, AOM is the most common

reason for prescription of antibiotics among children (2). Data from the National Ambulatory Medical Care Surveys and the National Hospital Ambulatory Medical Care Surveys indicate that 5.18 million episodes of AOM occur annually in the United States, costing approximately $2.98 billion (including direct and indirect costs) (2).

S. pneumoniae has been the pathogen most frequently isolated from middle ear fluid, identified in 30-60% of cases worldwide (34, 53). Pneumococcal otitis media (OM) is also recognized to be the most pathogenic and least likely to resolve spontaneously without antibiotic intervention, compared to other etiologies of OM (64). Pneumococcal OM is also more likely to be associated with complications, such as tympanic membrane rupture and spontaneous otorrhea (121). Like other manifestations of pneumococcal disease, infants and young children are at greatest risk for pneumococcal AOM, which has a peak incidence in the 6-18 month age group (38). While the pathogenesis of OM is not clear, multiple factors may converge and result in this youngest age group carrying the greatest burden of AOM disease, including anatomic abnormalities resulting in Eustachian tube dysfunction, preceding or co-infection with other viral and bacterial respiratory pathogens, environmental situations (such as crowding and daycare attendance), as well as immature or impaired immunologic status (133). A recent comprehensive review of the serotypes causing pneumococcal AOM worldwide showed that serotypes 6A, 6B, 14, 19A, 19F, and 23F were the most common serotypes causing AOM worldwide; serotypes 1, 5, and 7F, which cause serious invasive disease in some countries but are hardly ever found in colonization studies, were rarely associated with OM (112).

Due to the clinical significance and high economic burden caused by AOM, prevention of this illness through vaccination has been sought. There has been particular emphasis on the prevention of pneumococcal AOM with vaccination, since the development and licensure of the heptavalent pneumococcal conjugate vaccine (PCV7). The introduction of PCV7 into the routine childhood immunization schedule in the United States has been associated with a dramatic reduction in the incidence of invasive pneumococcal disease, while a more modest reduction in the cases of AOM among vaccine recipients (~5-10%) has been observed (38, 147). Data from the post-PCV7 era in the United States indicate that there has been an overall decrease in the number of visits to physicians for AOM and insertion of pressure equalizing tubes after the introduction of PCV7 (112). However, there has been an increase in the proportion of AOM caused by non-vaccine serotypes (particularly serotypes 3, 6A, and 19A), and by *H. influenzae* and *Moraxella catarrhalis* in vaccinated children (59, 114). In a Finnish study of the effect of PCV7 against AOM, episodes of AOM (all causes) were reduced by 6%, with a 34% reduction in AOM caused by pneumococci of any serotype, and a 57% reduction in cases caused by vaccine serotypes. There was a 33% increase in cases of AOM caused by non-PCV7 serotypes, and an increase in the cases of AOM caused by *Haemophilus influenzae* (40). Thus, replacement AOM among PCV7 recipients seems to be occurring more rapidly than replacement invasive disease, most likely because pneumococci colonizing the nasopharynx can easily gain access to the tympanic cavity through the Eustachian tube. The effect of PCV7 and other future multivalent PCVs on the incidence of AOM will probably be modest at best.

The importance of asymptomatic colonization

Healthy children commonly carry at least one serotype of *S. pneumoniae* in their nasopharynx without developing disease (90). Asymptomatic carriage of *S. pneumoniae* is central to the organism's transmission, and colonization is frequently followed by horizontal spread of the pneumococci through an individual's immediate environment and the surrounding community (15). It is known that pneumococcal disease will not occur without preceding nasopharyngeal colonization with the homologous strain, although the precise mechanism(s) by which colonizing *S. pneumoniae* travels to other anatomical sites and becomes pathogenic is poorly understood (15, 90).

The epidemiology of *S. pneumoniae* carriage differs between developed and developing nations, primarily due to differences in crowding, family size, socioeconomic status, underlying immune compromise and chronic conditions, and antibiotic usage. Based on a longitudinal carriage study of children in the United States, Gray, et al., reported the mean age of acquisition of the first *S. pneumoniae* serotype to be six months; 95% of children were colonized at some point during the first two years of life, and 73% had acquired at least two serotypes during the first 24 months, usually on different occasions (46). Two or three serotypes were present simultaneously in 4% and 0.3% of specimens, respectively. The duration of carriage was found to be serotype dependent and was between 3-5 months. A Finnish cohort investigation of healthy children and adolescents 1-19 years old yielded a peak carriage prevalence at approximately three years of age (~55% colonized), followed by a steady decline until a stable carriage prevalence of ~8% was achieved by about 10 years of age (15). Another Finnish study of the frequency of nasopharyngeal carriage in children 2-24 months old demonstrated an

increase in carriage prevalence from 13% for children less than six months old to 43% in children more than 19 months of age (126). The prevalence of carriage increased to 22-45% during respiratory infections, lending support to the hypothesis of increased pneumococcal adherence during viral infections.

Limited data exist documenting the prevalence of *S. pneumoniae* carriage among children in less developed nations. It is known that carriage occurs shortly after birth, the prevalence of carriage is much higher, and the prevalence of simultaneous colonization with multiple serotypes is greater. The prevalence of carriage nears 95% among healthy children less than three years old in developing nations, and simultaneous carriage of up to four serotypes for several months has been documented (90). This high frequency of multiple carriage among children in less developed countries and the dynamics and competition of species and serotypes colonizing the nasopharynx are particularly salient issues, given the potential for the pneumococcal conjugate vaccines to alter the balance between vaccine and non-vaccine serotypes within the nasopharynx.

Age-related immunity to pneumococcal colonization

The decline in colonization with increasing age and the observations about the contributions of different age groups to household-level pneumococcal colonization point toward the fundamental importance of understanding age-related immunity to pneumococcal colonization and the role of different age groups in pneumococcal epidemiology. It has been assumed that acquired immunity plays a role in modulating the carriage of pneumococci and preventing invasive disease, although there are little direct epidemiologic data to support this hypothesis. The increase and subsequent

decrease in pneumococcal carriage that has been observed in individuals 0-4 years in numerous studies is consistent with acquired immunity playing a role in reducing carriage. Specifically, it has been thought that the immune response to pneumococci in naturally exposed, unvaccinated children depends primarily on antibody directed against the polysaccharide capsule. This assumption is supported by the fact that immunization with polysaccharide conjugate vaccines generates an effective serotype-specific antibody response, treatment of infected patients with serum containing type-specific anti-capsular antibodies can aid in bacterial clearance, and patients with agammaglobulinemia are at an increased risk for pneumococcal disease (142).

However, despite the suggestion that anticapsular antibodies play a role in the development of immunity to pneumococci, there is little evidence showing that antibodies are the primary mechanism of naturally acquired immunity, and several studies have suggested otherwise (142). A few studies have shown a homotypic anticapsular serum antibody response to colonizing pneumococcal serotypes, but this response was only detectable in a proportion of the carriers (47-49). A few larger studies conducted in Finland demonstrated that serum antibodies to pneumococcal capsular polysaccharides appeared in response to carriage, but the response varied by individual serotype (109, 119, 120, 122). Furthermore, serum antibody responses to pneumococcal surface protein A (PspA), pneumococcal surface adhesion protein A (PsaA), and pneumolysin (Ply) elicited by colonizing pneumococci have been measured (109). It has also been demonstrated that salivary IgG and secretory IgA to PspA, PsaA, Ply, and pneumococcal capsular polysaccharide antigens were produced in response to carried pneumococci (119, 120). In mice, acquired protection against pneumococcal carriage is

not type specific and does not require antibodies but does depend on an effective $CD4^+$ T cell response (78, 135). The only direct evidence for acquired immunity playing a role in preventing acquisition and reducing colonization stems from a recent human challenge study (81). In this study, antibodies to PspA and choline-binding protein A (CbpA) increased on successful challenge, but no antibody response to lipoteichoic acid, IgA1 protease, Ply, proteinase maturation protein A, or PsaA was detected. The protective effect was associated with antibodies directed at the hypervariable N terminus of PspA, and no protective effect was detectable for pre-existing antibody to the homotypic capsular polysaccharide of the challenge strain (81). Thus, the demonstration of an increase and sharp decline in the frequency of pneumococcal colonization with increasing age suggests there is a process of naturally acquired immunity to pneumococci in unvaccinated individuals. Whether this adaptive immunity is anticapsular antibody-based or due to other mechanisms remains for future studies to resolve.

Nontypeable *S. pneumoniae*

Although infrequently found as the cause of invasive disease, nonencapsulated or nontypeable (NT) pneumococci are often found in colonization studies and have been identified as the cause of large outbreaks of conjunctivitis among healthy young people (17, 22, 50, 79, 107). NT pneumococci have also been shown to cause sporadic cases of conjunctivitis and occasionally cause acute otitis media in young children (9, 98, 118, 146).

NT pneumococci may arise via the following mechanisms: 1) downregulation of capsular expression; 2) failure to produce a capsule due to disruption in the *cps* region,

which contains the genes encoding the enzymes responsible for capsule biosynthesis; or 3) expression of a capsule that has not yet been identified. NT pneumococci have historically been defined as isolates that are optochin sensitive, soluble in 2% or 10% deoxycholate (bile salts), and fail to react with serotype-specific pneumococcal antisera (Quellung reaction). Study of NT pneumococci has been hampered by difficulty distinguishing true, unencapsulated pneumococci from closely related streptococci from the mitis group which also inhabit the oropharynx and nasopharynx. Optochin resistant pneumococci are regularly reported in the literature, and bile-insoluble pneumococcal isolates have been documented as well (146). *S. pseudopneumoniae*, which does not possess a pneumococcal capsule, are optochin resistant when grown in CO_2 but are optochin susceptible when grown in ambient air, and are bile insoluble, has recently been recognized (8). The existence of optochin-susceptible viridans group streptococci further compound this problem.

To address this identification issue, some investigators in clinical laboratories have added the AccuProbe® Gen-Probe® pneumococcus culture test (Gen-Probe, Inc., San Diego, CA) to the identification of NT pneumococci (35). The Gen-Probe® pneumococcus culture test is a DNA probe hybridization test based on the rRNA gene sequence. Sensitivity and specificity testing showed the Gen-Probe® pneumococcus test correctly identified 100% of *S. pneumoniae* isolates (AccuProbe® Gen-Probe® package insert). However, since there is less than 1% difference in the 16S rRNA genes of *S. mitis* (11 bp) and *S. oralis* (14 bp) and *S. pneumoniae*, some investigators have questioned the ability of this test to truly distinguish between these species (21). Carvalho, et al, demonstrated by DNA-DNA reassociation experiments that isolates of

suspected NT pneumococci from conjunctivitis outbreaks in New York, New Jersey, Illinois, New Hampshire, and California that were all optochin sensitive, bile soluble, and AccuProbe® positive were true NT pneumococci (21). Based on these results, the authors concluded that the AccuProbe® pneumococcal test is appropriate for identifying NT pneumococci, when the isolates originate from conjunctivitis outbreaks and give typical results for optochin and bile testing. The accuracy of the AccuProbe® pneumococcal test when it is applied to nonsterile site (i.e., nasopharynx or oropharynx) or sterile site isolates is unknown.

Little is known about the epidemiology or population genetics of NT *Streptococcus pneumoniae*; much of what is known about NT pneumococci stems from descriptions of conjunctivitis outbreaks and a limited number of community investigations. The association between nontypeable pneumococci and conjunctivitis was first postulated by Finland and Barnes in 1977 in a retrospective study of the incidence of capsular types in a Boston hospital from 1935-1974 (42). In 1982, Shayegani, et al., examined nontypeable pneumococcal isolates that caused outbreaks of conjunctivitis among college students in New York State during 1980, and compared them to nontypeable pneumococcal isolates from other conjunctivitis outbreaks that occurred among Marine Corp recruits in California in 1980, among college students in Illinois in 1980, and among a different group of university students in New York State in 1981 (116). Based on biochemical, immunological, and antibiotic sensitivity testing, and passage through mice, the authors concluded that the NT pneumococci that caused conjunctivitis outbreaks across the United States during 1980-1981 were antigenically

more similar to each other than to the tested encapsulated strains. They suggested that these nation-wide outbreaks were caused by NT pneumococci that were closely related.

In 1998, Medeiros and colleagues reported an outbreak of conjunctivitis that occurred from September 1994-September 1996 among 92 children 2-7 years old in Brazil (83). Approximately 40% of conjunctivitis cases were caused by *S. pneumoniae*, and 51% of the pneumococcal isolates were nontypeable.

Martin, et al, described a large outbreak of conjunctivitis caused by a strain of nontypeable *S. pneumoniae* that occurred on the Dartmouth College campus in 2002 (79). Among the 5,060 students enrolled at Dartmouth during the winter term of 2002, 698 (13.8%) were diagnosed with conjunctivitis between January 1 and April 12. Conjunctival swabs were obtained from 254 students, and 43.3% of the swabs grew bacteria that were identified as *S. pneumoniae*. Additional investigation of a subset of the isolates by the CDC confirmed the bacteria were pneumococci; however, none of the isolates could be serotyped, and capsular staining revealed that the bacteria lacked a polysaccharide capsule. Pulse-field gel electrophoresis (PFGE) showed two distinct but closely related banding patterns, and multilocus sequence typing (MLST) indicated a single sequence type (ST448) which had been reported only once before in the MLST database. The 16S rRNA was 99.4% similar to the corresponding sequence of TIGR4, the reference strain of *S. pneumoniae* whose sequence is listed in GenBank. The pneumococci isolated in the Dartmouth outbreak were compared to isolates from previous conjunctivitis outbreaks in New York and California in 1980 and in Illinois in 1981. Capsular staining of these isolates from prior outbreaks also indicated that these bacteria lacked a polysaccharide capsule, and the PFGE patterns of the isolates from New

York and California were identical to the Dartmouth isolates. The authors concluded that a strain of unencapsulated *S. pneumoniae* had caused this large conjunctivitis outbreak on the Dartmouth campus, and this strain was identical to another unencapsulated pneumococcal strain that caused conjunctivitis outbreaks among college students in New York and California in 1980, but was different from the unencapsulated pneumococcus that caused a conjunctivitis outbreak among college students in Illinois in 1981.

Following the Dartmouth conjunctivitis outbreak in January-April 2002, an outbreak of conjunctivitis was reported among school age children in Westbrook, Maine, from September 20-December 6, 2002 (22). Twenty-eight percent of 361 children from kindergarten, first, and second grades in the index elementary school had at least one episode of conjunctivitis during this period, and 11 of 20 (55%) students tested had culture-confirmed pneumococcal conjunctivitis. Three classroom teachers (13%) and three other school staff (15%) also had conjunctivitis during this period. Family members who did not attend the index school were surveyed, and 5% of 709 individuals reported conjunctivitis, with 76% of the household member conjunctivitis cases being contacts of students who were previously ill. Additionally, school nurses and child care staff from four schools and three child care centers in the community surrounding the index school reported 77 cases of conjunctivitis during the same time period. Among the 53 students with conjunctivitis at other schools, 19% had a family member at the index school, and 29% of 24 sick children at child care centers also had a sibling at the index school. Twenty conjunctival specimens were collected from sick students at the index school, and 15 samples were collected from students at schools outside of the index school; fifty-five percent and 33% of these samples, respectively, grew *S. pneumoniae*.

Nine pneumococcal isolates were sent to the U.S. Centers for Disease Control and Prevention for serotyping, and eight of the isolates could not be typed. One isolate obtained from a sick child at the index school was a serotype 38. The nontypeable isolates, but not the serotype 38 isolate, produced a PFGE pattern identical to the nontypeable pneumococcal isolates that caused the conjunctivitis outbreak on the Dartmouth campus earlier that year. The authors concluded that the Maine outbreak was caused by the same strain of nontypeable *S. pneumoniae* that caused the Dartmouth outbreak. While nontypeable pneumococci had previously been shown to cause outbreaks of conjunctivitis among college students, military recruits, and other sporadic cases of conjunctivitis, this report was the first documentation of an outbreak of nontypeable pneumococcal conjunctivitis among young children with transmission to persons in the community outside of the school setting.

Finally, Buck, et al, described a widespread community outbreak of nontypeable pneumococcal conjunctivitis that occurred from September 1-December 12, 2003, in two nearby cities in Minnesota (17). Seven hundred thirty-five cases of conjunctivitis were reported among adults and children, and 51% of the cases were reported from schools, childcare settings, and colleges. Unlike prior outbreaks of pneumococcal conjunctivitis, this outbreak appeared to spread quickly among members of the community, because a large proportion of cases had no known connections to the schools, childcare facilities, or colleges affected in the outbreak. Interviews with culture-confirmed cases did not reveal any additional shared community areas, e.g., fitness centers, churches, community centers, swimming pools, or employers (17). Forty-nine percent of conjunctival cultures (71 of 144) were positive for *S. pneumoniae*, and all isolates were nontypeable by

serotyping. PFGE identified three clonal groups, with 84% of the isolates belonging to one clonal group. MLST indicated the isolates had the same multilocus sequence type as the isolates obtained in the 1980 New York outbreak and the 2002 Dartmouth and Maine outbreaks. The authors suggest that the genetic relatedness of these isolates and their association with large outbreaks may indicate that this strain of nontypeable pneumococcus may have attributes favoring rapid and widespread transmission as a conjunctivitis pathogen (17).

A limited number of studies have attempted to describe the epidemiology or population structure of nontypeable pneumococci outside of the outbreak setting. Berron (2005) employed PFGE and MLST to describe the genetic structure of nontypeable pneumococci isolated from sporadic conjunctivitis cases in Spain from 1997-2002. Their aim was to determine whether the population structure of nontypeable pneumococci isolated from conjunctivas is similar to that of typeable pneumococci isolated from conjunctivas, and to establish the genetic relatedness of nontypeable pneumococci isolated from conjunctivas vs. other anatomic sites (12). The authors examined 75 pneumococci isolated from conjunctivas (40 nontypeable and 35 typeable) and 30 isolated from other anatomic sources (15 nontypeable and 15 typeable). The nontypeable and typeable conjunctival strains grouped in separate clusters, whereas nontypeable and typeable pneumococci isolated from other anatomic sources were similarly distributed. Nontypeable conjunctival strains belonged to two well-differentiated clonal lineages. The first lineage was represented by three newly described sequence types (ST941, ST942, and ST943), contained fully antibiotic susceptible strains, and appeared to be characteristic of conjunctival tissue. The second lineage was represented by the

previously described ST344, had a pattern of antibiotic resistance to penicillin, tetracycline, and erythromycin, and shared a genetic background with some nontypeable strains isolated from other sources (i.e., not just conjunctival tissue) (12). The authors hypothesized that the nontypeable strains belonging to the two clonal lineages described in their study might represent Spanish endemic pneumococcal clones involved in conjunctivitis cases over a long period of time, since several other nontypeable pneumococci isolated from conjunctivas in Spain in the 1980s also belonged to the same clonal lineages.

Hathaway, et al., evaluated 1,980 noninvasive and 215 invasive pneumococcal isolates for capsule production (51). The noninvasive isolates originated from nationwide surveillance for nasopharyngeal pneumococcal isolates in 1998, 1999, 2001, and 2002, in Switzerland. The invasive isolates were obtained from a nationwide collection of all invasive pneumococcal isolates in 1998 and 1999 in Switzerland. Twenty-seven isolates (1.2% of all isolates) were nonencapsulated; seven of the nonencapsulated isolates came from invasive disease contexts, and the remaining 20 isolates were colonizing strains. MLST and analysis of the capsular locus was used to divide the nonencapsulated strains into two groups: one group appeared to be closely related to encapsulated strains, as they had typical capsule genes and generated the MLST patterns of typeable strains. The other group appeared to be less closely related to known encapsulated pneumococci, as determined by MLST, and contained a sequence with homology to the *aliB* gene, instead of the capsule genes. All of the nonencapsulated strains in this second grouping contained the *aliB* homologue, and a subset of these strains contained another *aliB* homologue (*aliB*-like ORF1) upstream from the originally-described homologue (*aliB*-

like ORF2). BLAST analysis suggested the *aliB*-like ORF2 may have been acquired via horizontal gene transfer from *S. mitis*, and *aliB*-like ORF1 may have originated from *S. gordonii*. While the function of the putative AliB-like molecules is not known, the true AliB has been described as a membrane-bound lipoprotein which binds oligopeptides to deliver them to the Ami permease ABC transporter for uptake into the cell. In addition to taking up peptide for nutrition, it has been proposed that this mechanism may be a way for sensing the environment and that this ABC transporter may affect a gene regulator that controls pathways involved in diverse processes, such as induction of competence or adherence (51). The isolates in this second grouping of nonencapsulated pneumococci also include two clones that are geographically widespread and have caused conjunctivitis outbreaks and invasive disease. The origin of these nonencapsulated pneumococci that have *aliB*-like ORFs in the capsule locus is unknown, but the authors postulate that these strains may have derived from an encapsulated strain some time ago, based on finding a small region with homology to the *capN* gene of serotypes 33F and 37 downstream of the *aliB*-like ORF2. Altogether, the authors conclude that two groupings of strains among noninvasive and invasive nonencapsulated pneumococci could be identified: one group that is closely related to encapsulated strains and one group that does not appear to be closely related to encapsulated strains but may have originated from encapsulated pneumococci some time ago. Loss of capsule among this second grouping of nonencapsulated pneumococci was also associated with importation of one or two *aliB* homologues. While nonencapsulated pneumococci are usually considered to be avirulent, the examined strains were from both invasive and noninvasive contexts, and two identified clones have caused conjunctivitis epidemics worldwide.

In 2006, Hanage and colleagues examined 121 untypeable pneumococci obtained from nasopharyngeal swabs and middle ear fluid of Finnish children, and they employed a multilocus sequence approach to distinguish genuine nontypeable pneumococci from other closely related nontypeable streptococci (50). Using this approach, the authors determined that 70 of the 121 untypeable pneumococci in their dataset were true, nontypeable pneumococci; nineteen of the 70 pneumococci had the conserved capsular gene *cpsA* and therefore could be assigned a serotype (these isolates were characterized as nontypeable due to downregulation of capsular expression), and three isolates had major disruptions in their capsular region and could not produce a capsule. The remaining 48 isolates were a lineage of true, nontypeable pneumococci that have lost their capsular locus. The authors found that strains of this relatively old pneumococcal lineage have spread intercontinentally and have been isolated from carriage, mucosal, and invasive disease contexts. This lineage of nontypeable pneumococci has also been associated with outbreaks of conjunctivitis in the United States (specifically the Dartmouth, Maine, and Minnesota outbreaks, as well as the outbreaks from the 1980s). Hanage, et al., concluded that nontypeable pneumococci are pervasive components of carried populations, that they can arise naturally through downregulation, disruption, or loss of the *cps* locus, and that such variants can become successful and persist, becoming geographically widespread and causing disease.

In 2006, Shouval, et al., examined the relationship between nasopharyngeal carriage and disease rates of pneumococcal serotypes in specific anatomic sites, focusing on serotype-specific disease potential. The authors reported that nontypeable *S. pneumoniae* was an important cause of sporadic cases of acute conjunctivitis in southern

Israel between 2000 and 2004. Nontypeable pneumococci were the most common pneumococcal type causing conjunctivitis, but they were rarely found as the cause of invasive disease or acute otitis media (118). In a follow-up investigation of the nontypeable isolates that caused conjunctivitis in Israel in 2000-2004, Porat (2006) and colleagues used PFGE and MLST to characterize the population genetic structure of these isolates and commented on their relatedness to nontypeable clones isolated in Spain and the United States from patients with acute conjunctivitis (107). The authors demonstrated that the nontypeable pneumococci responsible for conjunctivitis in Israel grouped with two major clusters of pneumococci, one being a single locus variant of ST448 and the other was related to ST344. Both types of pneumococci appeared to be members of lineages that have lost capsular loci and have high rates of resistance to antimicrobial agents. Porat and colleagues hypothesized that loss of capsular genes and perhaps other, currently unidentified, genetic factors may provide nontypeable *S. pneumoniae* with a selective advantage in conjunctivitis.

In summary, bacterial isolates that form gray to green, α-hemolytic colonies on blood agar and are optochin sensitive, bile soluble, AccuProbe® Gen-Probe® pneumococcus culture test positive, but do not react with pneumococcal antisera appear to be genuine nontypeable pneumococci (although numerous variants undoubtedly exist). NT pneumococci may arise via downregulation of capsular expression, disruption in capsular genes that prevents capsule production, or loss of capsular loci. NT pneumococci rarely cause invasive disease, but are commonly associated with sporadic cases of conjunctivitis, as well as large conjunctivitis outbreaks among students, military recruits, and healthy community-dwelling individuals. A limited number of

investigations of the population genetic structure of NTs revealed the existence of at least two sub-groupings of NT pneumococci (as defined by MLST): one more homogenous group that appears to be closely related to typeable pneumococci, and another more heterogeneous group that has lost the capsular biosynthetic genes, may be distantly related to typeable pneumococci, and appears to exhibit a particular tropism for conjunctival cells. NT pneumococci also exhibit increased adherence to respiratory epithelial cells, suggesting that expression of little or no capsule may be advantageous in colonization or conjunctivitis (i.e., there may be tissue-specific advantages to decreased or lack of capsular expression) (60). Weiser and Kapoor also demonstrated that nonencapsulated pneumococci have increased transformability compared to encapsulated strains, suggesting that expression of capsular polysaccharide inhibits competence for genetic transformation (145).

While some knowledge of the nontypeable segment of the pneumococcal population exists, it is evident that this understanding is in its infancy. While assumed to be mainly avirulent, NT pneumococci have caused invasive disease and commonly cause conjunctivitis. Furthermore, certain NT pneumococci have been shown to be highly antibiotic resistant; this observation, coupled with an increased transformability and the fact that no currently available pneumococcal vaccines target the nontypeables, make the NT pneumococcal population worthy of further study and epidemiologic description.

Antibiotic resistance among *S. pneumoniae*

S. pneumoniae resistance to penicillin was first recognized in the 1960s, and, since then, penicillin-resistant pneumococci (PRP) have disseminated globally and

continued to increase in their degree of resistance (115). Subsequent global increase in macrolide drugs as first-line therapy for community-acquired pneumonia has lead to increased resistance of *S. pneumoniae* to this class of drugs, and surveillance studies from developed nations demonstrate that a large percentage of pneumococci are now macrolide resistant (65). The emergence of resistance to other classes of antimicrobials, such as fluoroquinolones, and the correlation between penicillin resistance and resistance to other drug classes has been recently recognized (23, 30, 148).

Selective pressure for drug-resistance predominantly stems from inappropriate antimicrobial exposure; for example, gradual restructuring of pneumococcal penicillin-binding proteins (PBPs) to a low-binding affinity version occurred because of continued exposure to β-lactam antibiotics (30). Pneumococci and other bacterial species that commonly reside in the nasopharynx (e.g., *Haemophilus influenzae* and *Moraxella catarrhalis*) may exchange resistance genes via recombination events and (less commonly for *S. pneumoniae*) plasmid transfer while co-residing in their ecological niche; individual antimicrobial usage creates an environment where the drug-resistant variants can establish and out-survive their drug-susceptible equivalents. Decreased carriage of drug-susceptible pneumococci within individuals can lead to increased transmission of resistant pneumococci at the community or population level. Expansion and worldwide spread of a few highly successful and multi-drug resistant (MDR, i.e., resistant to three or more classes of antibiotics) clones of *S. pneumoniae* have also contributed to increasing pneumococcal resistance (e.g., Spain^{23F} -1 clone is resistant to penicillin, chloramphenicol, tetracycline, and erythromycin and has been isolated in the United States, Europe, South and Central America, South Africa, and East Asia) (5, 94).

Additional MDR and globally distributed *S. pneumoniae* include clones within serotypes: 3, 6A, 6B, 9N, 9V, 14, 19A, and 19F (94).

Antimicrobial resistant pneumococci are a particular problem in developing countries, where the sale and use of antibiotics is largely unregulated and the simultaneous carriage of multiple pneumococcal serotypes in children has been documented. Due to a general absence of surveillance for pneumococcal serotypes and resistance profiles in less developed nations, an assessment of the extent of antimicrobial resistance among *S. pneumoniae* must be based upon limited point prevalence data and hospital-based drug reports (93, 94). Overall trends that can be gleaned include that drug resistant and MDR *S. pneumoniae* are highly prevalent and that resistance is increasing. Resistance to penicillin, macrolides, and co-trimoxazole has increased, and resistance to tetracycline or chloramphenicol has fluctuated widely (94). Increased pneumococcal resistance to individual drugs has led to a growing percentage of pneumococcal strains exhibiting resistance to many or all of the antimicrobials, and resistance to fluoroquinolones has emerged (94). Importantly, resistance to penicillin and erythromycin is particularly a problem in Asia, which has one of the highest prevalences of pneumococcal resistance in the world.

Antibiotic resistant *S. pneumoniae* in Vietnam

Injudicious use of antibiotics is a common public health problem in many Asian countries, including Vietnam, where antibiotic resistance and MDR among *S. pneumoniae* have been increasing at an alarming rate (108). In 2000, Larsson, et al., conducted a survey of 200 children 1-5 years old in a community in Bavi, Vietnam, to

examine patterns of antimicrobial use and antimicrobial susceptibility of respiratory tract pathogens (69). The authors found that 82% of the surveyed children had at least one symptom of ARI in the preceding four weeks, and, of these, 91% were treated with antibiotics. Approximately 50% of the children were found to carry *S. pneumoniae*, either in their nasopharynx or throat. Of the tested isolates, approximately 90% of *S. pneumoniae* were resistant (MIC >1 mg/L) to at least one antibiotic (88% resistant to tetracycline, 32% resistant to trimethoprim-sulphonamide, 25% resistant to chloramphenicol, and 23% resistant to erythromycin). On average, isolates of *S. pneumoniae* were reported to be resistant to two different classes of antibiotics.

Also in 2000, Parry, et al., conducted a multi-site carriage study of 911 urban and rural Vietnamese children (96). The authors reported that 44% of children were nasal carriers of *S. pneumoniae*. Thirty-four percent of nasal isolates had intermediate susceptibility to penicillin (MIC 0.1-1 mg/L), while 19% demonstrated full resistance (MIC >1 mg/L). Thirteen percent of *S. pneumoniae* isolates had intermediate susceptibility to ceftriaxone, and 1% of isolates were resistant. Penicillin resistance was more common in urban than rural children. More than 40% of isolates from urban children were also resistant to erythromycin, trimethoprim-sulfamethoxazole, chloramphenicol, and tetracycline. MDR was exhibited in 39% of *S. pneumoniae* isolates with intermediate resistance to penicillin and in 86% of penicillin resistant isolates. Parry and colleagues estimated that 50% of fully penicillin-resistant isolates were serotype 23, and these isolates often demonstrated MDR.

In an examination of nasal carriage of antibiotic-resistant pneumococci among children less than 5 years old in 11 countries in Asia, the Asian Network for Surveillance

of Resistant Pathogens (ANSORP) found a carriage proportion of 22% (across all sites, n=1105 isolates), 36% of which were reported to be penicillin nonsusceptible (70). The prevalence of penicillin nonsusceptibility in Vietnam was 70%. Penicillin resistance was related to residence in urban areas, enrollment in daycare, and a history of otitis media. The authors hypothesized that the spread of penicillin resistant *S. pneumoniae* that is carried by children in Asia may be due to the spread of a few predominant clones (e.g., Spain^{23F} -1).

In 2002, Bogaert, et al., reported their findings from a molecular epidemiologic study of pneumococcal carriage among children with upper respiratory tract infections in Hanoi, Vietnam (16). Fifty-two percent (n=84 isolates) of the strains demonstrated intermediate resistance to penicillin, 87% were intermediately resistant to co-trimoxazole, 39% were intermediately resistant to cefotaxime, 76% were fully resistant to tetracycline, and 73% were fully resistant to erythromycin. Seventy-five percent of isolates were MDR. Genotyping yielded two large clusters within the isolates, corresponding to the internationally-spreading MDR clone Spain^{23F}-1 and Taiwan^{19F}-14, respectively; the remaining isolates appeared to be Vietnam-specific.

In addition to carriage studies, a limited number of investigations into the resistance patterns of invasive *S. pneumoniae* have been conducted in Vietnam (97, 123, 124). Parry, et al., conducted surveillance for IPD from 1993-2002 at the Centre for Tropical Diseases in Ho Chi Minh City, Vietnam. From 1993-1995, penicillin nonsusceptible *S. pneumoniae* (MIC $\geq$0.1 mg/L) was isolated from the blood or cerebrospinal fluid (CSF) of 8% of patients (n=24, children and adults); this percentage of nonsusceptible isolates increased to 56% (n=36) during 1999-2002. Moreover, 28% of

isolates from patients with IPD were found to be fully penicillin resistant (here, defined as ≥2.0 mg/L). Multilocus sequence typing (MLST) revealed that 86% of the invasive penicillin-resistant isolates tested (n=14) were of the Spain^{23F} -1 clone.

In a study to characterize the macrolide resistance mechanisms among clinical isolates of *S. pneumoniae* obtained from children and adults in 10 Asian countries from1998-2001, the ANSORP Study Group reported that Vietnam had the highest prevalence of erythromycin resistance (88%, n=60 isolates, MIC ≥1.0 mg/L) compared to all study sites. Most of these isolates belonged to serotype 19F and had a MLST pattern similar to the Taiwan^{19F} -14 clone. Finally, the ANSROP Study Group also examined the *in vitro* susceptibility patterns of 685 clinical *S. pneumoniae* isolates collected from 14 centers in 11 Asian countries from 2000-2001. Isolates from Vietnam (n=64) demonstrated the highest prevalence of penicillin resistance (71%) compared to all participating country sites. Approximately 92% of Vietnamese isolates were also resistant to erythromycin. MLST showed that the majority of resistant *S. pneumoniae* isolates belonged to either the Taiwan^{19F} clone or the Spain^{23F} clone.

Although a few carriage studies and a limited number of clinical investigations of resistant pneumococci have been conducted in Vietnam, no investigation has explored the serotype distribution and antibiotic susceptibility patterns of invasive *S. pneumoniae* exclusively among children less than five years of age in Vietnam. Furthermore, risk factors for IPD due to nonsusceptible pneumococci have not been fully elucidated. It is against a background of highly resistant pneumococci that a serotype-specific conjugate vaccine may be introduced. The population-level effects of introducing a vaccine into this setting are unknown.

The pneumococcal conjugate vaccines (PCVs)

The currently available polysaccharide pneumococcal vaccine (PPV23) contains capsular polysaccharide of 23 pneumococcal serotypes and, while being relatively inexpensive, is weakly immunogenic in children less than two years old. The polysaccharide elicits a T-cell independent immune response, which is poorly developed in infants and young children and does not allow for a high-level antibody response and the induction of immunologic memory. Furthermore, the PPV23 has no effect on nasopharyngeal carriage of pneumococci or on otitis media (11, 91, 100, 101). The development of 11-, 9-, and 7-valent conjugate pneumococcal vaccines, in which the T-cell independent polysaccharide is made T-cell dependent via covalent linkage of the polysaccharide with a carrier protein, has provided the potential for protection against pneumococcal disease in the age group that is most at risk for invasive disease (100).

In 2000, a heptavalent pneumococcal conjugate vaccine (PCV) was licensed in the United States (Prevnar®) and is a formulation of the seven most prevalent pneumococcal capsular types in the United States (4, 6B, 9V, 14, 18C, 19F, and 23F) conjugated to a nontoxic variant of the diphtheria toxin. The most prevalent penicillin-resistant pneumococcal strains are also of the serotypes that are included in the conjugate vaccine. Post-licensure surveillance and epidemiologic studies have demonstrated that the conjugate vaccine is safe, immunogenic, highly effective in preventing invasive disease in infants and young children, effective in reducing otitis media and (to a lesser extent) pneumonia, and may be reducing pneumococcal disease in adults and unvaccinated children exposed to vaccinated children (herd immunity effect) (11, 14, 28,

31, 87-89, 91, 100, 101, 117, 147). Unlike the polysaccharide vaccine, the conjugate vaccine reduces carriage by preventing new nasopharyngeal acquisition of vaccine serotypes and, occasionally, of those serotypes that are cross-reactive to those in the vaccine. Consequently, the PCV reduces the prevalence of asymptomatic carriage of vaccine-type pneumococci, which is essential to reducing person-to-person transmission of *S. pneumoniae* (30, 87).

Concomitant with reductions in carriage and disease due to vaccine-type *S. pneumoniae*, an increase in colonization with non-vaccine serotypes (i.e., serotype replacement) has been observed in most studies that have examined pneumococcal carriage post-vaccination (28, 29, 31, 32, 80, 92, 140, 150). If pneumococcal strains compete with one another within their ecological niche, then reducing the carriage of seven vaccine serotypes and their immunologically related types would be anticipated to increase the carriage of non-vaccine serotypes. The impact of serotype replacement will depend largely on if non-vaccine strains are pathogenic and lead to replacement disease (30).

Owing to a lack of field data, mathematical models have been utilized to explore the serotype replacement phenomenon and have predicted the predominance of non-vaccine serotypes after the introduction of conjugate pneumococcal vaccines into populations under certain conditions (73, 74, 151). Mathematical models have also predicted that the degree of serotype replacement will be greatest when vaccine coverage is high. In fact, recent data from the United States suggest that colonization with non-vaccine serotypes are increasing following introduction of the conjugate vaccine, and, moreover, nonsusceptible, non-vaccine serogroups, such as serogroup 35, are emerging

(131, 132, 149). If the phenomenon observed in the United States is accurate, then introduction of the conjugate vaccine into a population characterized by an extremely high proportion of resistant *S. pneumoniae* (such as Vietnam) may have important and unexpected public health consequences.

Potential effect of PCVs on antibiotic resistant pneumococci

One of the most critical questions that remain is the extent to which the pneumococcal conjugate vaccine will reduce the prevalence of resistant pneumococci. There are several potential interactions between host immunity (either naturally acquired or vaccine-induced that selects for non-vaccine serotypes) and antibiotics that select for resistant pneumococci (30). Recent sequencing of the pneumococcal genome revealed that the genetic locus that encodes for the capsular biosynthetic enzymes (which determine serotype) are closely linked to the loci encoding penicillin and cephalosporin resistance. Further, Trzcinski, Thompson, and Lipsitch demonstrated that a susceptible pneumococcus exposed to DNA from a penicillin resistant strain of a different serotype, and selected with penicillin or cefotaxime, can acquire not only the resistance alleles, but also the new serotype, due to co-transformation of the *cps* locus (determines capsular serotype) with one or both *pbp* genes (encodes for penicillin binding proteins) (30). Such selection of both new capsular type and drug resistance in the face of selective pressure from antimicrobial agents allows for the possibility that selection by host immunity might also result in changes in both prevalent serotypes and antimicrobial resistance profiles. All studies that have examined the effect of conjugate vaccines on the carriage of antibiotic resistant pneumococci have demonstrated a reduction in carriage of resistant

vaccine strains after vaccination. Post-licensure data from the United States shows a dramatic reduction in the amount of IPD caused by vaccine-related penicillin nonsusceptible, macrolide resistant, and dually penicillin and macrolide resistant pneumococcal strains after vaccination with the heptavalent conjugate vaccine. However, these encouraging results may be curtailed to the extent that replacement with resistant non-vaccine types occurs. Examples that such replacement appears to be occurring include in the United States, where invasive disease due to a nonsusceptible, non-vaccine serotype (35B) may be emerging, and in Israel where resistant and non-vaccine serotypes 15B/C, 21, 33F, and 35B are causing acute otitis media (10, 106). Furthermore, recent findings have shown that some penicillin nonsusceptible *S. pneumoniae* clones are derived from capsular switch of known vaccine serotypes (30). Consequently, current declines in antimicrobial resistant pneumococcal disease caused by vaccine strains may change direction if population-level antibiotic pressure is not reduced.

Summary

Invasive pneumococcal disease (IPD) leads to millions of deaths and long-term neurologic complications annually in children under five years. Antibiotic resistant and multi-drug resistant (MDR) pneumococci are increasing, due to injudicious antibiotic usage and the global spread of MDR pneumococcal clones. Antibiotic resistance is a particular problem in Vietnam, which has one of the greatest rates of penicillin and macrolide resistance of all Asian countries. Vietnamese policymakers are considering introducing a pneumococcal conjugate vaccine into their pediatric population, but they

have no epidemiologic data upon which to make such decisions about the necessity of a relatively expensive vaccine. The impact of introducing a serotype specific vaccine into a population that is characterized by a potentially high proportion of multi-serotype carriage and extremely high prevalence of resistance are unknown. To address these vital questions about pneumococcal epidemiology and the appropriateness of a conjugate vaccine, we conducted a cross-sectional survey of pneumococcal colonization among household members of all ages in Nha Trang, Vietnam. We examined the prevalent serotypes and antimicrobial susceptibility patterns of pneumococci carried by individuals from all age groups. The results and implications of this carriage study conducted among family members in Vietnam are presented in the following chapters of this dissertation.

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Chapter 3

Serotype Distribution and Antibiotic Susceptibility of *Streptococcus pneumoniae* among Healthy Children and Adults in Nha Trang, Vietnam: Implications for Population Vaccination

Abstract

Background: The success of a pneumococcal conjugate vaccine (PCV), including direct and indirect effects, will depend on the proportion of individuals of all ages who are colonized, the pre-vaccine distribution of PCV and non-PCV serotypes carried by all age groups, the frequency of antibiotic resistance among PCV and non-PCV serotypes, and social mixing patterns. This investigation is the first report of the prevalence of colonization and antibiotic susceptibility distribution of PCV and non-PCV serotypes among healthy children, adolescents, and adults in Vietnam. It is also one of the limited number of surveys of pneumococcal colonization across all ages in any geographic location that included adolescents and adults.

Methods: We conducted a cross-sectional study of nasopharyngeal (NP) carriage in Khanh Hoa Province, Vietnam, in October 2006. Trained interviewers recruited 519 healthy participants from 115 households in one hamlet of Vinh Thanh commune. One NP swab specimen was collected from each household member. Pneumococci were identified using standard microbiologic procedures, and capsular typing was done with a multiplex PCR approach and conventional serotyping. Minimum inhibitory concentrations for 14 antibiotics were determined using the Vitek® 2 system.

Results: Forty percent of children ≤ 5 years, 12% of children 6-17 years, 3% of adults in household contact with children, and 1% of adults not in household contact with children were colonized with *S. pneumoniae*. Children ≤ 2 years were more likely to carry PCV serotypes, while older children and adults more commonly carried non-PCV serotypes and nontypeable pneumococci. Antibiotic resistance was high among all colonizing pneumococci, especially for penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole, and 66% of isolates were multidrug resistant.

Conclusions: PCV10 or PCV13 would target 79% of encapsulated pneumococci carried by young children in Vietnam. Older children and adults may serve as a source of replacement serotypes following PCV introduction. Use of PCV in young children may reduce the prevalence of resistant pneumococci. However, non-PCV serotypes and nontypeable pneumococci are resistant to various antibiotics may serve as a reservoir of resistance determinants in pneumococci.

Introduction

Streptococcus pneumoniae (the pneumococcus) is a respiratory pathogen that asymptomatically colonizes the human nasopharynx and can cause a range of mucosal and invasive disease. Although the global burden of pneumococcal disease is poorly defined, *S. pneumoniae* is responsible for an estimated 1-2 million deaths annually, and up to 1 million deaths among children less than five years, particularly among children in low-income nations (21, 26). In 2000, a seven-valent pneumococcal conjugate vaccine (PCV7, Prevnar®) was licensed in the United States for routine use in children 2-23 months of age and among children up to five years who are at high risk for pneumococcal

disease. Use of PCV7 in the United States led to a dramatic reduction in the incidence of invasive pneumococcal disease (IPD) caused by vaccine-targeted serotypes (36). This decline in IPD was observed among vaccine recipients, as well as non-vaccinated child and adult contacts of vaccinees, because PCV7 also reduces colonization with vaccine-targeted serotypes by 50-60%. Thus, use of PCV7 in young children created a herd effect by reducing transmission of vaccine serotypes and led to a population-level decrease in IPD. PCV7 also reduced the incidence of antibiotic-resistant pneumococcal disease in vaccinees and some non-vaccinated children and adults, because the serotypes included in PCV7 are frequently antibiotic resistant (18). Concomitant with reductions in carriage and disease due to vaccine serotypes, introduction of PCV7 has been associated with an increase in non-vaccine serotypes (serotype replacement). This increase in non-vaccine serotypes has been observed in all studies that examined colonization and in most disease studies. Although the increase in non-vaccine disease still remains small compared to the benefits gained by PCV7, the serotype replacement phenomenon may threaten the long-term utility of this vaccine.

Due to the overall success of PCV7 in the United States, other countries are now eager to introduce a pneumococcal conjugate vaccine (PCV) into their pediatric populations, and many private and public entities have partnered to provide financial assistance and accelerate the introduction of PCVs globally, particularly in resource-poor nations. Additional PCVs with expanded serotype coverage are available or will be brought to market shortly. A 10-valent PCV (PCV10, Synflorix™), which includes serotypes 1, 5, and 7F, in addition to the serotypes included in PCV7, is approved for use in Canada and Australia and was recently authorized for use in Europe. A 13-valent PCV

(PCV13, covers PCV7 serotypes +1, 3, 5, 6A, 7F, and 19A) is currently in global phase III clinical trials, and regulatory filings are anticipated in 2010. The rational choice of a PCV for a particular country should be based on knowledge of the pneumococcal disease burden and serotypes causing IPD in a particular geographic location.

Vietnam, in particular, is eligible for financial support for a PCV through the GAVI Alliance and has expressed interest in introducing a PCV in young children. However, there is limited information on the disease burden and distribution of pneumococcal serotypes causing disease in Vietnam. Anh and colleagues recently showed that *S. pneumoniae* is a common cause of invasive bacterial disease in hospitalized children in Nha Trang, Vietnam. This study provided a much-needed estimate of the incidence of IPD in children. Among children < 5 years who lived in Nha Trang, Vietnam, there were 48.7 cases per 100,000 children per year (95% CI: 27.9-85.1 per 100,000 children); among children < 1 year, the incidence rate was 193.4 per 100,000 children per year (95% CI: 97.1-384.9 per 100,000 children). However, no serotype information was available from this study (2). A few investigations have described serogroups or serotypes causing upper respiratory tract infections or colonizing young children in Vietnam; these studies showed that pneumococcal colonization is common among young children and is often associated with otitis media, but there is no information on the frequency of colonization or the serotypes carried by older children, adolescents, and adults (5, 20, 29, 32-34). Data from older age groups are vital, since these individuals may be a source of replacement strains in a population; knowing the serotypes carried by older age groups can also help anticipate what, if any, benefit older, non-vaccinated individuals may gain from exposure to vaccinated children. Furthermore,

because serotypes targeted by the PCVs are commonly antibiotic resistant, introduction of a vaccine could provide a much-needed reduction in resistant pneumococci in Vietnam, which has one of the highest rates of β-lactam and macrolide resistance of all Asian countries.

The success of a PCV in Vietnam (including direct and indirect effects and the rate at which serotype replacement may occur) will depend on the percent of individuals of all ages who are colonized with pneumococci, the pre-vaccine distribution of PCV and non-PCV serotypes carried by all age groups, the frequency of antibiotic resistance among PCV and non-PCV serotypes, and social mixing patterns. Such data can also motivate predictions about the potential long-term effect of a PCV on serotype replacement and declines in antibiotic resistance. Here, we describe the frequency of pneumococcal colonization, the proportion of PCV and non-PCV serotypes, and the frequency of antibiotic resistance among pneumococci carried by children, adolescents, and adults living in a single hamlet in Nha Trang, Vietnam. This investigation is the first study in Vietnam to report the frequency of pneumococcal colonization among all age groups. It is also one of the limited number of surveys of pneumococcal colonization across all ages in any geographic location and the only one from an Asian country that included adolescents and adults.

Methods

Study design

We conducted a cross-sectional study of nasopharyngeal (NP) carriage in Phu Vinh hamlet of Vinh Thanh commune, Nha Trang district, Vietnam, in October, 2006.

We randomly selected households in Phu Vinh with at least one child ≤ 5 years and, separately, households with only adults (≥ 18 years) from a detailed census list that was compiled by the Khanh Hoa Provincial Health Service prior to this carriage study. Trained interviewers recruited households until 75 households with children and 40 households with adults were enrolled. All currently residing household members were invited to participate in a one-time NP swab procedure and survey interview. A total of 146 households were approached, and 115 households participated (79%). Ninety-six households with children were approached, and 75 households agreed to participate (78%). Fifty households containing only adults were approached, and forty agreed to participate (80%). The most common reason for refusal was that all household members were not available to participate, because they were employed far from home and had to travel extensively during the day.

All NP swab procedures took place at the Vinh Thanh Commune Health Center. At the time of the swab procedures, three households which previously contained only adults presented with a small child (i.e., an adult female household member had given birth in the time between recruitment and survey). These three households were considered to contain one child ≤ 5 years for purposes of analysis. Altogether, 78 households contained at least one child ≤ 5 years, and 37 households contained only adults (≥ 18 years).

Trained physicians obtained a single NP swab specimen from all participants, in accordance with World Health Organization recommendations (27). NP samples were obtained with calcium alginate-tipped wire swabs (Calgiswab® Type 1, Puritan Medical Products Company LLC, Guilford, ME). Swabs were passed through the anterior nares

to the back of the nasopharynx, left in place for approximately two seconds, and rotated 180 degrees before removal. After collection, all NP swab specimens were immediately inoculated into STGG transport media and held at 4°C for no more than four hours at the Commune Health Center. The NP swabs were then transported to the local hospital, where they were stored at -20°C for one week. NP swabs were shipped on dry ice to the University of Michigan and stored at -80°C for one month prior to analysis (27).

Informed consent was obtained from all adults (≥ 18 years) and parents of children prior to participating in the investigation. Oral assent was also obtained from participants 6-17 years old prior to the NP swab procedure. All study procedures were approved by the Health Sciences Institutional Review Board (IRB) at the University of Michigan (HUM00006257), the IRB of the National Institute of Hygiene and Epidemiology in Hanoi, Vietnam, and the IRB of the International Vaccine Institute in Seoul, Korea (2006-009).

Bacterial culture and isolation

Frozen vials of STGG medium containing NP swab specimens were warmed in a 37°C water bath for 20 minutes and then vortexed for 20 seconds. Thawed samples in media (50 µl) were inoculated on trypticase soy agar with 5% sheep blood (TSA II) containing gentamicin (2.5 mg/L) and separately on non-selective media (TSA II). All specimens were incubated at 37°C in 5% CO_2 overnight (O/N). Presumptive identification of pneumococcal colonies was based on colony morphology and α-hemolysis on blood agar (27). Up to 10 pneumococcal colonies were sampled per

individual. If more than one pneumococcal colony morphology was present, then up to 10 colonies per morphology type were isolated.

Confirmatory identification of pneumococci was based on optochin sensitivity (zone ≥14 mm with 6mm P discs after O/N incubation at 37°C in 5% CO_2), solubility in 10% deoxycholate using the plate method, and Gram stain (27).

Capsular typing

DNA was extracted from each pneumococcal isolate as previously described, and the Latin American formulation of a sequential multiplex PCR was used for serotyping (13, 28). Each PCR reaction included *Streptococcus pneumoniae* ATCC® 49619 (serotype 19F) as a positive control and a no-template (dH_2O) negative control. PCR products were electrophoresed on 2% NuSieve® GTG® agarose gels in TAE buffer at 125-128 V for 38 minutes. Gels were stained in ethidium bromide (0.5 µg/mL), and PCR product sizes were compared to a 50-bp molecular standard (Novagen, Inc). A 20% random selection of PCR-designated serotypes was confirmed with latex agglutination followed by the Quellung reaction (Statens Serum Institut, Denmark). The presence of newly-identified serotype 6C was investigated among serotype 6A isolates by use of a multibead assay based on monoclonal antibodies and multiplex PCR (Moon Nahm, MD, personal communication) (37).

Nontypeable pneumococci

Any isolate that was optochin sensitive and bile soluble but could not be typed with either the multiplex PCR or antisera was considered to be nontypeable (NT). The

Gen-Probe® AccuProbe® pneumococcus culture identification test (Gen-Probe, Inc., San Diego, CA), a DNA probe hybridization test based on the rRNA gene sequence, was used to confirm the identity of all NT isolates (3, 8, 12).

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) for penicillin, cefotaxime, ceftriaxone, chloramphenicol, ertapenem, erythromycin, levofloxacin, linezolid, moxifloxacin, ofloxacin, telithromycin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin were determined using the AST-GP65 card for the Vitek® 2 system (bioMerieux, France) (1, 9, 14, 23). For susceptibility testing, frozen pneumococcal isolates were subcultured twice on TSA II and incubated for 20-24 hours at 37°C in 5% CO_2. *S. pneumoniae* ATCC® 49619 served as the quality control and was grown in ambient air, in accordance with Vitek® 2 system recommendations. MIC cut-points were defined according to the CLSI criteria (2007) (10). Multidrug resistance (MDR) was defined as resistance to three or more different classes of antibiotics.

Selection of unique pneumococcal strains

It was presumed that the multiple colonies isolated per colonized individual would most likely be the same strain. To test this assumption, we compared the serotypes and antibiograms of each pneumococcal isolate to the other co-colonizing isolates from the same individual. Co-colonizing pneumococci from the same individual were considered different if they had different serotypes (or, a typeable vs. nontypeable isolate) or the same type but different antibiograms. Antibiograms were considered

different if the MIC value of at least one antibiotic differed from the other pneumococci colonizing the same individual. MIC values were considered different for antibiotics if they diverged by more than one dilution factor above or below the MIC value, in line with standard clinical laboratory practice (4). One isolate was randomly selected for further analysis from each group of identical pneumococci colonizing an individual.

Frequency analyses

All frequency analyses were performed with SAS v 9.1.3 (SAS Institute, Cary, NC).

Results

Study sample

Five-hundred and nineteen individuals from 115 households in Phu Vinh hamlet participated in the survey and nasopharyngeal (NP) swab procedure (Figure 3.1). One hundred fifty-nine children and adolescents (< 18 years) were surveyed. Of individuals less than 18 years, 104 (65%) were ≤ 5 years, 22 (14%) were 6-10 years, and 33 (21%) were 11-17 years. Three-hundred and sixty adults (≥ 18 years) took part in the survey and NP swab procedure. Of adults, 142 (39%) were 18-30 years, 120 (33%) were 31-49 years, and 98 (27%) were 50 years and older (percentages do not add to 100 because of rounding). The youngest participant was one-and-a-half months old, and the oldest participant was 86 years old.

Carriage of *S. pneumoniae* by age

Fifty-six people (11% of all 519 individuals) were colonized with *S. pneumoniae*. Pneumococcal carriage was greatest among young children and decreased with increasing age (Figure 3.2). Overall, increasing age was associated with a decrease in the likelihood of pneumococcal colonization ($p<0.0001$). Among children ≤ 5 years, the frequency of colonization increased with age, peaked in three-year-olds, and then declined with further increases in age (Figure 3.2). Of all age groups, children who were three years old were most likely to be colonized with pneumococci (67%), and adults in household contact with young children who were 18-30 years old and adults not in household contact with children were least likely to be colonized (1%).

Description of unique pneumococcal strains for antibiotic susceptibility analyses

A total of 568 pneumococcal strains were isolated from 56 colonized individuals. Encapsulated pneumococci comprised 70.4% of the isolates (n=400), while 29.6% of isolates (n=168) were nontypeable (NT). The 400 encapsulated pneumococci were isolated from 39 individuals; of these isolates, 67 unique encapsulated strains were selected on the basis of their antibiograms for analysis. The 168 NT pneumococci were isolated from 20 individuals; of these isolates, 29 unique NT strains were selected on the basis of their antibiograms for analysis. That is, a total of 96 unique pneumococcal isolates (67 typeable and 29 NT) were obtained from 56 individuals and were used for the antibiotic susceptibility frequency analyses in this study.

An in-depth description of the 96 unique pneumococcal isolates chosen for the antibiotic susceptibility analyses is presented in Table 3.1. Of the 56 colonized individuals, four individuals (7%) carried two different serotypes or a typeable and a NT

strain (see colonized individuals nos. 12, 47, 48, and 51 in Table 3.1). These individuals with multi pneumococcal type carriage generated a total of 10 strains. The remaining 52 individuals were colonized with all the same serotype or all NT pneumococci. Of the 52 people colonized with all the same pneumococcal type, 27 individuals (52%) were colonized with pneumococci that had identical antibiograms, and one pneumococcal strain was randomly selected from the group of identical isolates for analysis (n=27 total strains). Sixteen individuals (31%) were colonized with two unique strains (n=32 total strains), and nine individuals (17%) were colonized with three unique strains (n=27 total strains). Among the 25 colonized individuals from whom ≥ 2 unique pneumococcal strains were isolated, the antibiotic(s) that served as the basis for the difference between the strains were as follows: erythromycin (68%), chloramphenicol (36%), tetracycline (16%), cefotaxime (20%), ceftriaxone (8%), ofloxacin (8%), penicillin (4%), and telithromycin (4%) (percentages sum to greater than 100 because multiple antibiotics served as the basis of the different between strains in 10 instances). In every case, designating two or more strains as unique based on the MIC value(s) of a particular antibiotic(s) resulted in capturing co-colonizing strains of the same type but a different resistance level (e.g., two strains of the same type from one person were considered different if one was susceptible to an antibiotic and the other strain was either intermediately or fully resistant to the antibiotic). Based on our definition of unique strains for the antibiotic susceptibility analyses, no isolates were designated as unique if they were the same type and had the same interpretation of their MIC value (i.e., all sensitive, intermediately, or fully resistant).

Serotype distribution of colonizing pneumococci

Among the 67 unique encapsulated pneumococci that were isolated from individuals of all ages, the serotype distribution was as follows: 14 (18%), 19F (16%), 23F (16%), 15B/C (15%), 6A (10%), 11A (10%), 6B (9%), 16F (3%), 34 (2%) (percents do not add to 100% because of rounding). No serotype 6C was discovered among the 6A carriage isolates (Moon Nahm, personal communication).

Colonization among young children 0-2 years was dominated by serotypes 19F, 14, 23F, and 6B (Table 3.2). Older age groups frequently carried types 6A, 15B/C, 11A, and 16F, in addition to types 19F and 23F. Individuals three years and older carried nontypeable pneumococci more commonly than the youngest age group.

Antibiotic susceptibility distribution

Based on the unique pneumococcal isolates (n=96), full antibiotic resistance was common among all colonizing pneumococci, particularly for penicillin (49%), chloramphenicol (16%), erythromycin (62%), tetracycline (76%), and trimethoprim-sulfamethoxazole (91%). Additionally, some pneumococci demonstrated intermediate levels of resistance to: penicillin (45%), cefotaxime (8%), ceftriaxone (1%), erythromycin (6%), ofloxacin (2%), telithromycin (1%), tetracycline (6%), and trimethoprim-sulfamethoxazole (5%). Isolates were fully sensitive to ertapenem, levofloxacin, linezolid, moxifloxacin, and vancomycin. Sixty-six percent of all pneumococci were resistant to three or more classes of antibiotics (multidrug resistant, MDR).

Antibiotic resistance by serotype

The frequency of antibiotic resistance differed by serotype, although resistance to penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole was common across most serotypes (Table 3.3). Serotypes found in the current study that are targeted by one of the pneumococcal conjugate vaccines (PCVs) (19F, 23F, 14, 6B, and 6A) were generally resistant to erythromycin, tetracycline, and trimethoprim-sulfamethoxazole; sixty-two percent of these serotypes were multidrug resistant. Serotypes found in this investigation that are not covered by one of the PCVs (15B/C, 11A, 16F, and 34) were also frequently resistant to erythromycin, tetracycline, and trimethoprim-sulfamethoxazole, and 50% of these serotypes were multidrug resistant.

Discussion

In order to make a rational choice about a pneumococcal conjugate vaccine (PCV) for young children in Vietnam and to predict how a PCV may alter the pneumococcal population, we examined the prevalence of colonization, the serotype distribution, and the antibiotic susceptibility patterns among pneumococci carried by healthy household members of all ages in Nha Trang. These data also further the current understanding of the epidemiology of *S. pneumoniae* by focusing on colonization among adolescents and adults, two segments of populations that have been largely ignored in colonization studies, even though these individuals (older adults in particular) suffer from pneumococcal disease and likely make a contribution to endemic transmission of pneumococci (25). In the present study, carriage was common among young children, peaked in 3 year-olds, and declined with increasing age. Children 0-2 years commonly

carried serotypes 19F, 14, 23F, and 6B. Colonization among adolescents and adults was low, and these age groups frequently carried non-PCV-targeted serotypes and nontypeable pneumococci. Resistance to penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole was high across all serotypes and was particularly great among serotypes carried by young children.

There was a dramatic difference between the serotype distributions observed among 0-2 year-olds and individuals three years and older. PCV-targeted serotypes clearly dominated as the colonizing types among the 0-2 year-olds, while individuals of all other ages carried a combination of PCV and non-PCV-targeted serotypes. The most highly colonized age group was three year-olds, who are usually not targeted for a PCV. Of the 23 pneumococci isolated from three year-olds, 43% of isolates were serotypes covered by a PCV (19F, 23F, 6B, 6A), while 57% of the isolates were serotypes not covered by a PCV (15B/C) or were nontypeable. This shift toward non-PCV serotypes with increasing age most likely indicates acquired immunity to the most prevalent capsular types, preventing or reducing reacquisition of these serotypes or greatly limiting the duration of carriage of these serotypes upon re-colonization. As a consequence, different age groups in a population will harbor different types and serotypes of pneumococci. These data call attention to the potential for older children and adults to serve as a source for replacement serotypes following PCV introduction. Children who are 3-5 years may make the largest contribution to replacement serotypes following introduction of a PCV among 0-2 year-olds, because these children are frequently colonized with non-PCV-targeted serotypes, may carry pneumococci longer than adolescents and adults because of a still-maturing immune response, and may have an

increased number of infectious contacts with young children compared to other age groups. Adolescents and adults can also make an important contribution to the changing pneumococcal population following PCV introduction, depending on the prevalence, duration, and density of colonization among older age groups, the number of infectious contacts between young and older age groups, whether acquired immunity to a limited number of serotypes provides any protection against all serotypes, and a number of bacterial factors (e.g., competitive ability) that impact the capacity of certain pneumococci to dominate over others. While it remains for future investigation to address these questions about the nature of immunity to colonization, the duration and density of carriage among different age groups, and pneumococcal characteristics that would enable one strain or strains to dominate over others, our observations of different serotypes carried by different age groups highlight the need for focusing on individuals of all ages in future colonization studies and any investigation that attempts to predict the shift in the pneumococcal population that may occur with introduction of a PCV among young children. Thus, when considering the source of replacement serotypes in a population, one must consider the non-PCV pneumococci carried by vaccinees, as well as those transmitted by unvaccinated, older age groups.

The prevalence of antibiotic resistance was high among all serotypes in our colonization study in Vietnam. Pneumococci were commonly resistant to penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole, and 66% of all isolates were multidrug resistant. Prior colonization studies in Vietnam have also documented high levels of resistance to penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole and multidrug resistance among pneumococci, although these studies

focused only on children (5, 20, 29, 32). The high levels of resistance demonstrated by pneumococci carried by all age groups most likely reflect antibiotic usage practices in Vietnam, the successful spread of certain multidrug resistant pandemic clones (e.g., Spain 23F-1 and Taiwan 19F-14), and the competitive advantage gained by these bacteria in an environment of strong selective pressure due to overuse of antibiotics. All bacteria exist in populations, and it is plausible that we captured a greater frequency of resistant than sensitive strains, assuming resistant strains can out-compete sensitive strains in the face of antibiotics. Some sensitive strains may still exist, perhaps at a very low prevalence. Because of the association between antibiotic resistance and serotypes included in the PCVs, introduction of a conjugate vaccine in Vietnam would be expected to reduce the prevalence of antibiotic resistance among serotypes targeted by the vaccine, perhaps shifting the pneumococcal population toward sensitive strains. However, the prevalence of high levels of resistance among non-PCV-targeted serotypes provides reason for reduced optimism about the effect of a conjugate vaccine on resistance in Vietnam. In the face of continuing selective pressure, it is conceivable that a PCV will reduce the prevalence of resistant serotypes targeted by the vaccine, allowing for resistant non-PCV serotype to become dominant.

The current investigation focused on pneumococcal carriage and not invasive disease; basing a vaccination policy decision on carriage and not invasive disease data presents certain challenges that merit discussion. For example, it is important to know that the colonizing serotypes are also the types causing invasive pneumococcal disease, and that the vaccine serotype formulation will match the distribution of disease-causing serotypes in a given country. It is also vital to know how much geographic and temporal

variation to expect in serotype distribution, in the absence of vaccination. On the basis of serotype, pneumococci display little antigenic variability within a single host, but exhibit extensive population-wide variation that is consistent in space and time (24). In general, the distribution of serotypes causing pneumococcal disease appears to change little over periods of at least four decades, but larger changes may occur over longer intervals (7). The main serogroups recovered from carriage or invasive or mucosal disease studies are also geographically consistent, although not identical, across continents (24). Serogroups 6, 14, and 19 are among the leading causes of invasive infection among all children in all regions of the world, while serotypes 1 and 5 appear to differ somewhat in the degree to which they cause invasive disease in various regions (7). Additionally, longitudinal carriage studies indicate that the serotypes dominating the population of colonizing pneumococci in one geographic area do not change dramatically over weeks-to-months, although the rank order of the most prevalent serotypes found at any one point in time may vary (15-17). This relative stability of pneumococcal serotypes over place and time indicates that a cross-sectional colonization study can provide adequate detail about the carried pneumococcal population that is most likely causing disease in a given location. Furthermore, it has long been observed that certain serotypes appear to be more "invasive" than others, because they are rarely isolated from colonized persons and only detected in invasive disease studies. Serotypes 1 and 5, which are responsible for a large amount of invasive disease in other Asian countries, were not found in this colonization study; these serotypes are also rarely isolated in carriage studies and are hypothesized to have an extremely short duration of carriage and a high invasive disease attack rate. Thus, while colonization is a prerequisite for the development of disease, and many of the

serotypes that cause pneumococcal disease in a community will be captured in a carriage study, focusing on colonization will likely miss the few serotypes that can cause serious disease.

The current investigation has certain additional limitations that warrant mention, as they may impact the inferences made here. First, the prevalence of colonization among adolescents and adults was low and prevented statistical examination of certain risk factors for carriage among these age groups. Given that no previous study in Vietnam reported colonization prevalence for adolescents and adults, we based our enrollment quotas for older age groups on the estimate of 10% of the population being colonized at any one point in time. The point prevalence of colonization among these age groups provided in this study can be used to refine sampling approaches in future studies. Second, we did not sample households that contained only older children and adolescents in household contact with adults. Future colonization studies should aim to include older children and adolescents not in household contact with young children to ascertain the contribution of this segment of the population to pneumococcal transmission. Third, it is possible that nasopharyngeal sampling alone may be inferior to combined nasopharyngeal and oropharyngeal sampling in adults and may have resulted in an underestimate of colonization among adults (22, 35). If feasible, future colonization studies should include larger numbers of adolescents and adults and incorporate both nasopharyngeal and oropharyngeal sampling for older age groups. Fourth, we sampled households from one peri-urban neighborhood in Vietnam. We selected Phu Vinh hamlet in Nha Trang District because it was comprised of households whose number of residents (i.e., household size) and the income level of those residents were close to the national

averages in Vietnam. This neighborhood was also chosen because it was an approximate closed sub-population, being geographically divided from the remaining three neighborhoods in the commune, and our goal was to capture pneumococci in a single transmission system. Additionally, only households with all members present on the day of the survey were allowed to participate, selecting out households with members who travel extensively for work or school. While it is certain that most residents of Phu Vinh travel into and out of the neighborhood to some degree for work, schooling, and healthcare, our focus on this one neighborhood and household selection criterion may have lead us to capture a reduced diversity of serotypes compared to other geographic areas where there is more in-and-out migration (e.g., larger cities, such as Ho Chi Minh City and Hanoi). The serotype distributions presented in our study may not be fully representative of the carried pneumococcal population in Vietnam. However, the main serotypes we found in Phu Vinh (i.e., serotypes 23F, 19F, 6B, 14, 15B/C) have also been reported among children from various geographic locations in Vietnam, including urban and rural sites (5, 19, 20, 29-34). Future studies of pneumococcal colonization should cover multiple geographic sites and focus on carriage among individuals in rural, semi-rural, and urban areas to more fully capture the diversity of carried serotypes in Vietnam.

Finally, we defined pneumococcal strains as being unique if pneumococci co-colonizing an individual had different serotypes (or, a typeable and a nontypeable) or if pneumococci co-colonizing an individual were all of the same type, but had different antibiograms. Antibiograms were considered different if the MIC value of at least one antibiotic differed from the other pneumococci colonizing the same individual. MIC values were considered different for antibiotics if they diverged by more than one

dilution factor above or below the MIC value, in line with standard clinical laboratory practice (4). Based on this definition, no co-colonizing isolates were designated as unique if they were the same type and had identical interpretations for the MIC values of all antibiotics (i.e., all sensitive, intermediately, or fully resistant). We employed this definition of unique strains in the frequency analysis of antibiotic resistance among our pneumococcal isolates, as opposed to basing our analysis on all 568 isolated pneumococci, which would have overstated the prevalence of resistance by counting identical isolates as separate. Our goal was to report the percent of pneumococci that were susceptible, intermediately, and fully resistant to 14 antibiotics in our sample. We selected multiple pneumococcal isolates per person because colonizing bacteria exist in populations, with some co-colonizing isolates harboring resistance determinants not held by others. We specifically intended to capture this diversity with our sampling procedure and definition of unique isolates, in an attempt to accurately show the degree of antibiotic resistance among pneumococci in Vietnam. It should be emphasized that our definition of "unique" is based on the phenotype of antibiogram and not the presence of the genes responsible for resistance. While it is possible that we labeled isogenic strains that only differ by the presence of a particular plasmid harboring a resistance gene as different, our intention was to capture the frequency of phenotypic resistance among the population of transmitted pneumococci in Vietnam. In fact, such misclassification of strains as different is likely common with our approach, since the antibiotics by which isolates were defined as different commonly included erythromycin, chloramphenicol, and tetracycline, resistance to which is borne on conjugative transposons or plasmids. Our definition of unique strains may be more appropriate in contexts that focus solely on penicillin

resistance, where phenotypic resistance is determined by alterations in penicillin binding proteins (a permanent structural change to the cell); thus, co-colonizing pneumococcal strains that are sensitive vs. resistant to penicillin may truly be considered different. While this definition of unique co-colonizing isolates is not appropriate for other analyses and in other contexts, it is appropriate for our particular intention of describing the frequency of phenotypic antibiotic resistance among colonizing pneumococci in Vietnam and hypothesizing how the prevalence of resistance may change with the introduction of a PCV.

Despite the acknowledged deficiencies of focusing on carriage data to inform vaccination policy, we can make a recommendation about the appropriateness of a PCV for young children in Vietnam based on the current study and information from a few other studies in Asia. The currently licensed 7-valent pneumococcal vaccine (PCV7) includes serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. PCV10 (approved for use in Europe) includes the serotypes in PCV7 plus serotypes 1, 5, 7F. The 13-valent pneumococcal conjugate vaccine (PCV13) that is currently in phase three trials includes the seven serotypes currently found in PCV7 and six additional serotypes (1, 3, 5, 6A, 7F and 19A). Considering encapsulated strains, PCV7 would target 79% of pneumococci carried by children ≤ 2 years; this percentage increases to 89% if cross-protection with 6A is assumed. Other non-vaccinated age groups also benefit from vaccination of small children with one of the PCVs, because of their impact on carriage and transmission. If all age groups are considered, PCV7 would target 59% of encapsulated pneumococci in Vietnam; that percentage increases to 69% if there is cross-protection with 6A. If encapsulated and nonencapsulated pneumococci are considered together, PCV7 could

target up to 49% of all pneumococci circulating among all age groups in Vietnam and up to 79% of all pneumococci transmitted among children ≤ 2 years. We did not find evidence of the three additional serotypes included in PCV10 (1, 5, 7F) among the carried pneumococcal population in Vietnam. However, as previously noted, serotypes 1 and 5 are recognized to have short durations of carriage, are typically not found in colonization studies, but cause a large amount of invasive disease in other Asian countries (6). Of the six additional serotypes in PCV13 (1, 3, 5, 6A, 7F and 19A), we found only serotype 6A in the present carriage study. The non-PCV7 serotypes recovered in the current study were 15B/C, 11A, 16F, and 34, none of which are targeted by any of the PCVs. Other non-PCV7 serotypes isolated from previous clinical or carriage studies in Vietnam included serotypes 23A, which is also not targeted by any of the PCVs (20, 29, 32, 34).

Based on our carriage data and combined with a limited number of carriage and invasive disease studies from Vietnam and other Asian countries, health policymakers in Vietnam should consider introducing PCV10 or PCV13 in young children. PCV10, in particular, could be considered because of its current availability. The benefit of waiting to introduce PCV13 into Vietnam's pediatric population would be negligible, especially if there is some cross-protection against serotype 6A provided by PCV7. While non-vaccinated adults may garner some protection from vaccination of young children with a PCV, the serotype distribution carried by adults seems to differ from those included in the conjugate vaccine. While the success of PCV7 in young children and its indirect effect in adults have also generated interest in using a PCV in elderly populations, any future health policy changes to also use a PCV in adult populations will need to consider

the relative proportions of PCV and non-PCV serotypes carried by these older individuals, as well as the frequency of colonization among older age groups (11).

This investigation is the first study in Vietnam to report the frequency of pneumococcal colonization among all age groups. It is also one of the limited number of surveys of pneumococcal colonization across all ages in any geographic location and the only one from an Asian country that included adolescents and adults. These pre-vaccine data give valuable baseline characteristics about the transmittable pneumococcal population in Vietnam and underscore the necessity of a pneumococcal vaccine among children. While not having perfect serotype distribution coverage for Vietnam, PCV10 is currently available and would target approximately 49% of all pneumococci circulating among children and adults and 79% of all pneumococci carried by young children, in addition to providing protection against the main serotypes causing serious invasive disease in most other parts of Asia. It is important to note that colonizing and disease-causing serotypes following introduction of a PCV will have to be monitored for evidence of replacement, given that 31-41% of pre-PCV serotypes in Vietnam are not covered by the vaccines. Protein-antigen based pneumococcal vaccines or multiserotype vaccines that prevent disease but not colonization (while still protecting infants) continue to be a public health priority.

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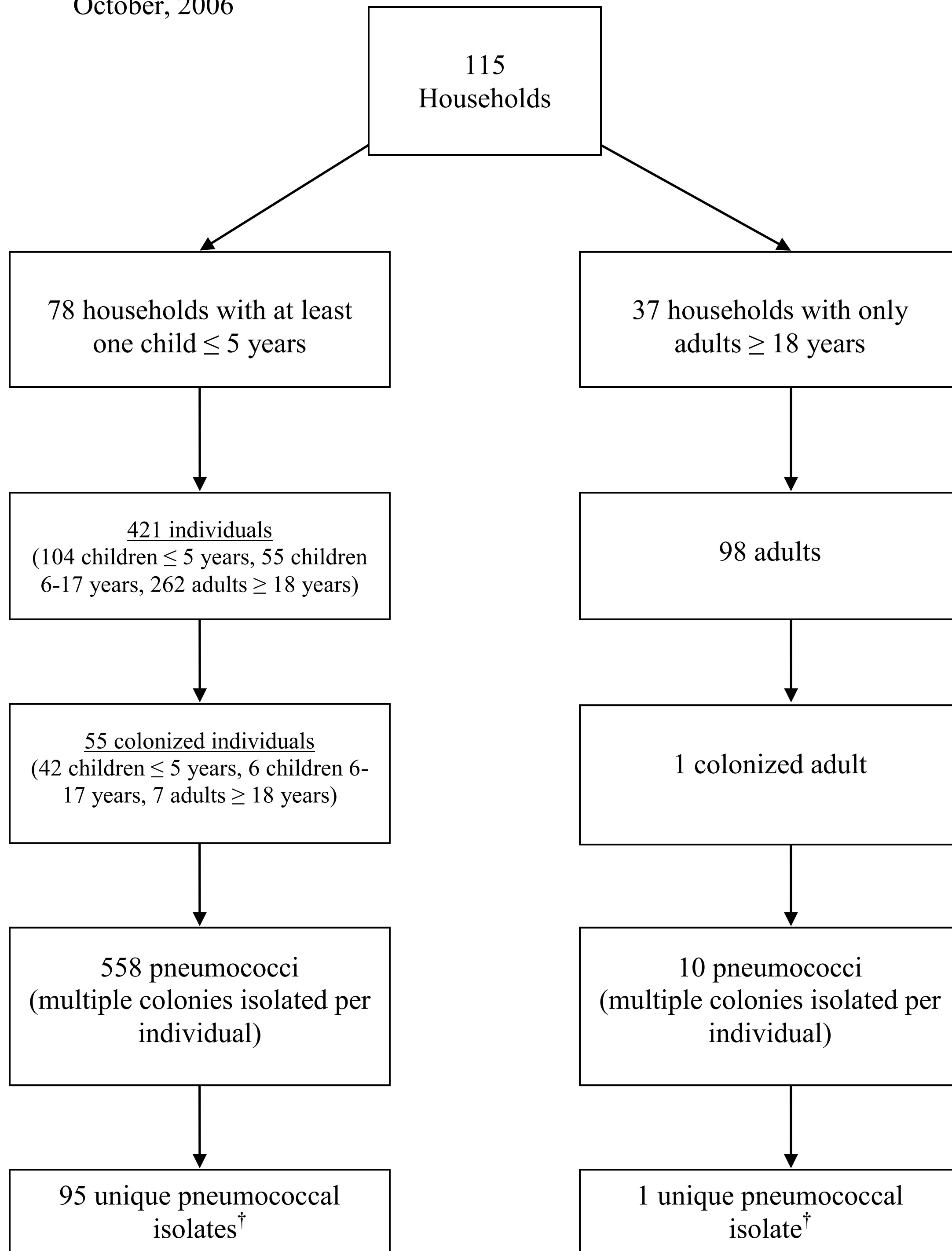
Figure 3.1: Number of households, individuals, colonized individuals, and pneumococcal strains isolated from household survey of pneumococcal carriage, Nha Trang, Vietnam, October, 2006



† Co-colonizing pneumococci from the same individual were considered different if they had different serotypes (or, a typeable vs. nontypeable isolate) or the same type but different antibiograms. Antibiograms were considered different if the MIC value of at least one antibiotic differed from the other pneumococci colonizing the same individual. MIC values were considered different for antibiotics if they diverged by more than one dilution factor above or below the MIC value.

Figure 3.2: Percent distribution of *Streptococcus pneumoniae* colonization by age. Five hundred and nineteen individuals participated in a household survey of colonization in Nha Trang, Vietnam, October, 2006. *S. pneumoniae* were isolated from nasopharyngeal swabs. The number of surveyed individuals in each age group is shown in parentheses below each corresponding bar. The percentages above each bar represent the percent of that age group that was colonized with *S. pneumoniae*.

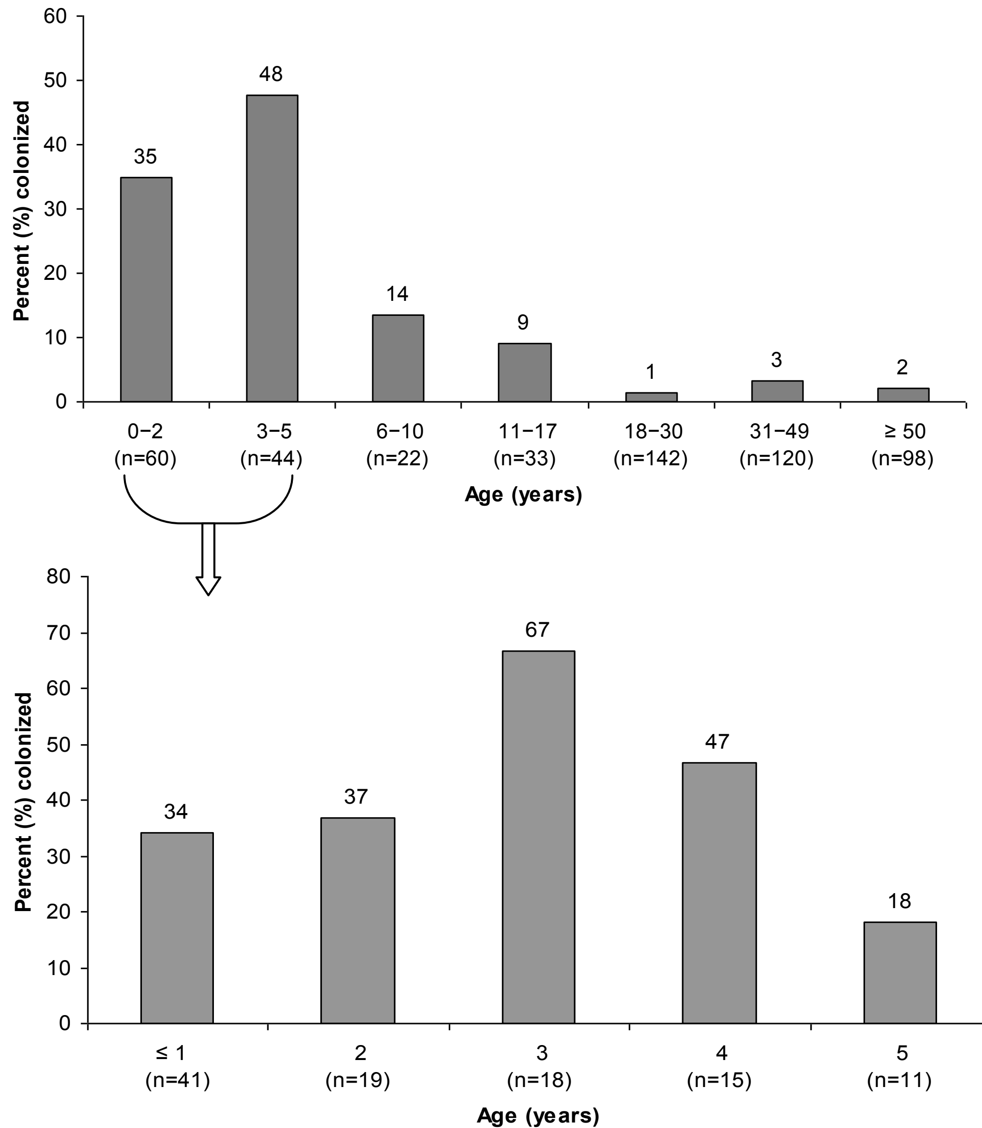


Table 3.1: Description of 96 unique pneumococcal strains isolated from 56 colonized individuals from a study of pneumococcal carriage among 519 healthy individuals living in households in Nha Trang, Vietnam, in October, 2006.

Strain No.	Unique strain ID	Colonized individual No.	Serotype or nontypeable (NT)	Antibiotic(s) by which antibiogram differs	No. of unique pneumococcal strains per colonized individual	Do isolates have different MIC interpretations (S/I/R)?
1	1430041084	1	6B	--	1	--
2	14300780915	2	14	Erythromycin	2	Yes (S vs. R)
3	1430078095		14			
4	1430078102	3	14	--	1	--
5	1430113102	4	6A	Chloramphenicol	2	Yes (S vs. R)
6	1430113103		6A			
7	1430115097	5	NT	--	1	--
8	1430117135	6	NT	--	1	--
9	1430154152	7	19F	Cefotaxime	2	Yes (S vs. I)
10	1430154151		19F			
11	1430173115	8	NT	--	1	--
12	1430205096	9	14	Telithromycin	2	Yes (S vs. I)
13	1430205099		14			
14	1430205102	10	23F	Chloramphenicol	2	Yes (S vs. R)
15	1430205106		23F			
16	1430217034	11	NT	--	1	--
17	1430217091	12	NT	--	2	--
18	1430217094		6B			
19	1430221077	13	11A	Erythromycin, Tetracycline	3	Yes for erythromycin (S vs. R) Yes for tetracycline (S vs. I vs. R)
20	1430221075		11A			
21	1430221074		11A			
22	1430241089	14	34	--	1	--
23	1430247063	15	19F	--	1	--
24	14302470710	16	19F	--	1	--
25	1430268061	17	11A	--	1	--
26	1430281145	18	19F	--	1	--
27	1430281155	19	NT	Cefotaxime, ceftriaxone, chloramphenicol, erythromycin	3	Yes for erythromycin (S vs. I vs. R) Yes for chloramphenicol (S vs. R vs. R) Yes for cefotaxime (S vs. S vs. I) Yes for ceftriaxone (S vs. S vs. I)
28	1430281157		NT			

Strain No.	Unique strain ID	Colonized individual No.	Serotype or nontypeable (NT)	Antibiotic(s) by which antibiogram differs	No. of unique pneumococcal strains per colonized individual	Do isolates have different MIC interpretations (S/I/R)?
29	1430281159		NT			
30	1430281167	20	NT	Cefotaxime, chloramphenicol	3	Yes for chloramphenicol (S vs. R vs. R) Yes for cefotaxime (S vs. I vs. S)
31	1430281162		NT			
32	1430281168		NT			
33	1430305071	21	NT	Chloramphenicol, erythromycin	2	Yes for erythromycin (S vs. R) Yes for chloramphenicol (S vs. R)
34	1430305072		NT			
35	1430327111	22	6B	Erythromycin	2	Yes (S vs. R)
36	1430327112		6B			
37	1430329093	23	14	--	1	--
38	1430329122	24	NT	Ceftriaxone	2	Yes (S vs. R)
39	1430329123		NT			
40	1430329135	25	6B	--	1	--
41	1430348031	26	NT	--	1	--
42	1430348111	27	23F	Erythromycin	2	Yes (S vs. R)
43	1430348112		23F			
44	1430348135	28	23F	Chloramphenicol	2	Yes (S vs. R)
45	1430348137		23F			
46	1430348151	29	23F	Penicillin, Chloramphenicol, erythromycin	3	Yes for erythromycin (S vs. S vs. I) Yes for chloramphenicol (S vs. S vs. R) Yes for penicillin (S vs. I vs. I)
47	1430348152		23F			
48	1430348159		23F			
49	1430358066	30	NT	--	1	--
50	1430359061	31	19F	Cefotaxime	2	Yes (S vs. I)
51	1430359063		19F			
52	1430359071	32	15B	Erythromycin, tetracycline	3	Yes for erythromycin (S vs. S vs. I) Yes for tetracycline (R vs. S vs. R)
53	1430359073		15B			
54	1430359075		15B			
55	1430364081	33	23F	--	1	--
56	1430377145	34	11A	Erythromycin,	3	Yes for

Strain No.	Unique strain ID	Colonized individual No.	Serotype or nontypeable (NT)	Antibiotic(s) by which antibiogram differs	No. of unique pneumococcal strains per colonized individual	Do isolates have different MIC interpretations (S/I/R)?
				tetracycline		erythromycin (S vs. S vs. R) Yes for tetracycline (S vs. R vs. R)
57	1430377149		11A			
58	14303771410		11A			
59	1430387018	35	NT	--	1	--
60	1431616047	36	19F	--	1	--
61	1431621043	37	6A	--	1	--
62	1431629013	38	14	Erythromycin	2	Yes (S vs. R)
63	1431629016		14			
64	1431629044	39	6A	--	1	--
65	1431632034	40	16F	Tetracycline	2	Yes (S vs. R)
66	1431632039		16F			
67	1431638047	41	23F	--	1	--
68	1432022045	42	NT	Chloramphenicol	2	Yes (S vs. R)
69	1432022049		NT			
70	1432033032	43	15C	Erythromycin, ofloxacin	3	Yes for erythromycin (S vs. R vs. R)
71	1432033033		15C			Yes for ofloxacin (S vs. I vs. S)
72	1432033038		15C			
73	1432048041	44	NT	Cefotaxime, erythromycin	3	Yes for erythromycin (S vs. R vs. R) Yes for cefotaxime (S vs. I vs. S)
74	1432048043		NT			
75	1432048045		NT			
76	1432048052	45	NT	--	1	--
77	1432076036	46	NT	--	1	--
78	1432076051	47	6A	Chloramphenicol	2	Yes (S vs. R)
79	1432076053		NT			
80	1432083036	48	19F	Erythromycin (among the 19Fs)	3	Yes (S vs. R)
81	1432083037		19F			
82	14320830316		6B			
83	1432086022	49	15B	Erythromycin, ofloxacin	3	Yes for erythromycin (S vs. I vs. R) Yes for ofloxacin (S vs. I vs. S)
84	1432086024		15B			
85	1432086029		15B			
86	1432086044	50	15B	--	1	--
87	1432115031	51	6A	Erythromycin (among the 6As)	3	Yes (S vs. R)

Strain No.	Unique strain ID	Colonized individual No.	Serotype or nontypeable (NT)	Antibiotic(s) by which antibiogram differs	No. of unique pneumococcal strains per colonized individual	Do isolates have different MIC interpretations (S/I/R)?
88	14321150310		6A			
89	14321150317		NT			
90	1432116043	52	19F	--	1	--
91	14321230310	53	NT	--	1	--
92	1432133038	54	NT	--	1	--
93	1432133044	55	14	Erythromycin	2	Yes (S vs. R)
94	1432133049		14			
95	1432133053	56	14	Erythromycin	2	Yes (S vs. R)
96	1432133054		14			

Table 3.2: Serotype distribution by age group. The distribution of 60 pneumococcal types carried by 56 healthy children and adults is shown below (Nha Trang, Vietnam, 2006).

Up to 10 pneumococcal colonies were selected per colonized individual. However, only four colonized individuals (7% of colonized people) carried two different serotypes or a typeable and a nontypeable pneumococcus. The percentages listed below represent the proportion of each age group that carried a particular serotype, based on examination of one serotype per person or two different types per individual, in the four instances of multi-type carriage.

	Serotype									
	PCV serotypes					Non-PCV serotypes and Nontypeables (NT)				
Age Group (years)	19F N (%)	23F N (%)	14 N (%)	6B N (%)	6A N (%)	15B/C N (%)	11A N (%)	16F N (%)	34 N (%)	NT N (%)
0-2 (n=21)†	5 (24)	3 (14)	4 (19)	4 (19)	2 (10)	1 (5)	0 (0)	0 (0)	0 (0)	4 (19)
3-5 (n=21)†	2 (10)	2 (10)	1 (5)	1 (5)	3 (14)	2 (10)	2 (10)	0 (0)	0 (0)	10 (48)
6-17 (n=6)‡	1 (17)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	1 (17)	0 (0)	3 (50)
≥ 18 (n=8)‡	0 (0)	1 (13)	2 (25)	0 (0)	0 (0)	1 (13)	0 (0)	0 (0)	1 (13)	3 (38)

†Row percentage sums to greater than 100% because two individuals carried two types of pneumococcus.
‡ Row percentage sums to greater than 100% due to rounding error.

Table 3.3: Antibiotic resistance by serotype. Pneumococci were isolated from nasopharyngeal swabs of 519 healthy household members in Nha Trang, Vietnam, in 2006. The percent of 96 unique[†] pneumococcal isolates that are fully resistant to select antibiotics is shown below.

	Antibiotic					
Serotype or Nontypeable (NT)	Benzylpenicillin N (%)	Chloramphenicol N (%)	Erythromycin N (%)	Tetracycline N (%)	Trimethoprim-Sulfamethoxazole N (%)	Resistant to ≥ 3 classes of antibiotics N (%)
19F (n=11)	10 (91)	0 (0)	10 (91)	9 (82)	11 (100)	10 (91)
23F (n=11)	1 (9)	5 (45)	1 (9)	0 (0)	11 (100)	1 (9)
14 (n=12)	5 (42)	0 (0)	7 (58)	11 (92)	12 (100)	9 (75)
6B (n=6)	1 (17)	0 (0)	5 (83)	6 (100)	4 (67)	4 (67)
6A (n=7)	0 (0)	4 (57)	6 (86)	6 (86)	7 (100)	5 (71)
15B/C (n=10)	6 (60)	0 (0)	3 (30)	9 (90)	10 (100)	6 (60)
11A (n=7)	7 (100)	0 (0)	3 (43)	4 (57)	7 (100)	4 (57)
16F (n=2)	0 (0)	0 (0)	0 (0)	1 (50)	2 (100)	0 (0)
34 (n=1)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)
NT (n=29)	17 (59)	6 (21)	23 (79)	26 (90)	25 (86)	24 (83)

[†]Co-colonizing pneumococci from the same individual were considered unique if they had different serotypes (or, a typeable vs. nontypeable isolate) or the same type but different antibiograms. Antibiograms were considered different if the MIC value of at least one antibiotic differed from the other pneumococci colonizing the same individual. MIC values were considered different for antibiotics if they diverged by more than one dilution factor above or below the MIC value.

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Chapter 4

High Prevalence of Presumptive Nontypeable *Streptococcus pneumoniae* Carried by Healthy Household Members in Vietnam

Abstract

Background: Nontypeable (NT) pneumococci have been associated with large outbreaks of conjunctivitis and can cause invasive disease among healthy people. Previous study of NTs has been plagued by difficulty distinguishing true NT pneumococci from closely related viridans streptococci. We recovered an usually high proportion of presumptive NT pneumococci from a colonization study conducted among healthy household members in Vietnam, and these isolates were highly drug resistant.

Methods: We conducted a cross-sectional study of nasopharyngeal carriage in Khanh Hoa Province, Vietnam, in October, 2006. NT pneumococci were defined as isolates that were α-hemolytic, optochin susceptible when grown in 5% CO_2, bile soluble, failed to react with antipneumococcal polysaccharide capsule antisera or be typed by multiplex PCR, and were Gen-Probe® AccuProbe® pneumococcus culture test positive. A subset of presumptive NTs was tested for the presence of *lytA* and *psaA*, and a different subset of NTs was tested for the presence of *cpsA*. Minimum inhibitory concentrations for 14 antibiotics were determined using the Vitek® 2 system.

Results: A total of 568 pneumococci were isolated, and 29.6% of isolates (n=168) were NT. All tested NT pneumococci were positive for *psaA*, while only two isolates were

positive for *lytA*. All tested NTs appeared to lack the *cps* locus. Thirty-three percent of colonized children (≤ 5 years), 50% of colonized older children and adolescents (6-17 years), and 42% of colonized adults (≥ 18 years) carried NT pneumococci. NT strains demonstrated full resistance to: penicillin (58.6%), ceftriaxone (3.5%), chloramphenicol (20.7%), erythromycin (79.3%), tetracycline (89.7%), and trimethoprim-sulfamethoxazole (86.2%). Eighty-three percent of NT pneumococci were multidrug resistant.

Conclusions: Our inclusive selection process of typical and less typical pneumococcal colony morphologies during culture may have lead us to capture a more accurate representation of the colonizing pneumococcal population, i.e., NTs may comprise a larger proportion of the colonizing pneumococcal population than previously recognized. Older children and adults may be more likely to carry NTs, potentially due to age-related immunity to encapsulated pneumococci. The NTs isolated in this study were highly antibiotic resistant and highlight the need to consider NT pneumococci in the development of vaccines that target all pneumococci.

Introduction

Streptococcus pneumoniae (the pneumococcus) remains a major public health pathogen, causing an estimated 1-2 million child deaths every year, globally (27). The pneumococcal polysaccharide capsule is considered a main virulence factor, functioning to reduce opsonophagocytosis by limiting access of phagocytes to complement bound to the cell wall (2). Although infrequently found as the cause of invasive disease, nonencapsulated or nontypeable (NT) pneumococci are often found in colonization

studies (approximately 2-10% of all carried pneumococci). NTs are assumed to be mainly avirulent; however, they have been identified as the cause of large outbreaks of conjunctivitis among healthy young people (7, 10, 18, 26, 36). NT pneumococci also cause sporadic cases of conjunctivitis and occasionally cause acute otitis media in young children (6, 34, 40, 43).

NT pneumococci may arise via the following mechanisms: 1) downregulation of capsular expression; 2) failure to produce a capsule due to disruption in the *cps* region, which contains the genes encoding the enzymes responsible for capsule biosynthesis; or 3) expression of a capsule that has not yet been identified. Study of NT pneumococci has been hampered by difficulty distinguishing true, unencapsulated pneumococci from closely related streptococcal species that also inhabit the oropharynx and nasopharynx. Historically, the identification of pneumococci has been based on four phenotypic tests that are used in diagnostic laboratories: colony morphology, optochin sensitivity, bile solubility, and agglutination with antipneumococcal polysaccharide capsule antibodies (Quellung reaction). However, there have been many observations of optochin-resistant pneumococci (22, 30, 35), and bile insoluble pneumococci also have been reported (15, 16, 28). The existence of optochin-susceptible viridans group streptococci further compound this problem. Furthermore, there have been numerous reports of oral streptococci harboring virulence-factor encoding genes that are usually associated with pneumococci, such as *lytA* (encodes for the major autolysin) and *ply* (encodes for pneumolysin, a pore-forming toxin) (21, 43). *S. pseudopneumoniae*, a new species of streptococci, has recently been recognized (4). *S. pseudopneumoniae* does not possess a capsule, is optochin resistant when grown in CO_2 but optochin susceptible when grown in

ambient air, and is bile insoluble (4). These identification problems are the source of considerable confusion in clinical laboratories and in the published literature (43).

One study in southern Israel examined 148 NT pneumococci isolated from children < 3 years old who were healthy carriers (n=87), children with acute otitis media (n=15), or children with acute conjunctivitis (n=46) and showed that NTs can be highly antibiotic resistant (36). This observation, coupled with an increased transformability and that no currently available pneumococcal vaccine targets the nontypeables, make the NT pneumococcal population worthy of further study and epidemiologic description. Here, we describe the prevalence of NT pneumococci carried by healthy household members of all ages in Vietnam prior to vaccine introduction. We defined NT pneumococci according to the classic phenotypic criteria, with the addition of the Gen-Probe® AccuProbe® pneumococcus culture test. We also tested a subset of our NT isolates for the presence of the capsular locus (*cpsA*) and *lytA* and *psaA*. Based on these criteria, we discovered an unusually high proportion of NTs among pneumococci isolated from this population; especially notable was a potential increase in the carriage of NT isolates with increasing age and the high degree of antibiotic resistance among these isolates. To our knowledge, this report is the first to describe carriage of nontypeable pneumococci among healthy individuals of all ages. We discuss the implications of these observations for vaccine design, the evolution of antibiotic resistance among naturally recombining pneumococci, and understanding the human immune response to polysaccharide capsule.

Methods

Study design

We conducted a cross-sectional study of nasopharyngeal (NP) carriage in Phu Vinh hamlet of Vinh Thanh commune, Nha Trang district, Vietnam, in October, 2006. We randomly selected households in Phu Vinh with at least one child ≤ 5 years and, separately, households with only adults (≥ 18 years) from a detailed census compiled by the Khanh Hoa Provincial Health Service prior to this carriage study. Trained interviewers recruited households until 75 households with children and 40 households with adults were enrolled. All currently residing household members were invited to participate in a one-time NP swab procedure and survey interview. A total of 146 households were approached, and 115 households participated (79%). Ninety-six households with children were approached, and 75 households agreed to participate (78%). Fifty households containing only adults were approached, and forty agreed to participate (80%). The most common reason for refusal was that all household members were not available to participate because they were employed far from home and had to travel extensively during the day.

All NP swab procedures and face-to-face interviews took place at the Vinh Thanh Commune Health Center. Trained interviewers asked adult participants about health behaviors, health history, hand hygiene practices, recent antibiotic usage for respiratory and non-respiratory illnesses, and demographics using standardized questionnaires. Adult caregivers were asked similar questions about their children, including the child's breastfeeding history, child or day-care attendance, and school attendance. At the time of the interviews and swab procedures, three households which previously contained only adults presented with a small child (i.e., an adult female household member had given

birth in the time between recruitment and survey). These three households were considered to contain one child ≤ 5 years for purposes of analysis. Altogether, 78 households contained at least one child ≤ 5 years, and 37 households contained only adults (≥ 18 years).

Trained physicians obtained a single NP swab specimen from all participants, in accordance with World Health Organization recommendations (31). NP samples were obtained with calcium alginate-tipped wire swabs (Calgiswab® Type 1, Puritan Medical Products Company LLC, Guilford, ME). Swabs were passed through the anterior nares to the back of the nasopharynx, left in place for approximately two seconds, and rotated 180 degrees before removal. After collection, all NP swab specimens were immediately inoculated into STGG transport media and held at 4°C for no more than four hours at the Commune Health Center. The NP swabs were then transported to the local hospital, where they were stored at -20°C for one week. NP swabs were shipped on dry ice to the University of Michigan and stored at -80°C for one month prior to analysis (31).

Informed consent was obtained from all adults (≥ 18 years) and parents of children prior to participating in the investigation. Oral assent was also obtained from participants 6-17 years old prior to the NP swab procedure. All study procedures were approved by the Health Sciences Institutional Review Board (IRB) at the University of Michigan (HUM00006257), the IRB of the National Institute of Hygiene and Epidemiology in Hanoi, Vietnam, and the IRB of the International Vaccine Institute in Seoul, Korea (2006-009).

Bacterial culture and isolation

Frozen vials of STGG medium containing NP swab specimens were warmed in a 37°C water bath for 20 minutes and then vortexed for 20 seconds. Thawed samples in media (50 µl) were inoculated on trypticase soy agar with 5% sheep blood (TSA II) containing gentamicin (2.5 mg/L) and separately on non-selective media (TSA II). All specimens were incubated at 37°C in 5% CO_2 overnight (O/N). Presumptive identification of pneumococcal colonies was based on colony morphology and α-hemolysis on blood agar (31). Up to 10 pneumococcal colonies were sampled per individual. If more than one pneumococcal colony morphology was present, then up to 10 colonies per morphology type were isolated.

Confirmatory identification of pneumococci was based on optochin sensitivity (zone ≥14 mm with 6mm P discs after O/N incubation at 37°C in 5% CO_2), solubility in 10% deoxycholate using the plate method, and Gram stain (31).

Capsular typing

DNA was extracted from each pneumococcal isolate as previously described, and the Latin American formulation of a sequential multiplex PCR was used for serotyping (14, 32). Each PCR reaction included *Streptococcus pneumoniae* ATCC® 49619 (serotype 19F) as a positive control and a no-template (dH_2O) negative control. PCR products were electrophoresed on 2% NuSieve® GTG® agarose gels in TAE buffer at 125-128 V for 38 minutes. Gels were stained in ethidium bromide (0.5 µg/mL), and PCR product sizes were compared to a 50-bp molecular standard (Novagen, Inc). A 20% random selection of PCR-designated serotypes was confirmed with latex agglutination followed by the Quellung reaction (Statens Serum Institut, Denmark).

Identification of nontypeable pneumococci

Any α-hemolytic isolate that was either optochin sensitive or bile soluble (possible atypical pneumococcus), or both optochin sensitive and bile soluble (possible typical pneumococcus), but could not be typed with either the multiplex PCR or antisera was considered a potential nontypeable (NT) pneumococcus. The AccuProbe® Gen-Probe® pneumococcus culture identification test (Gen-Probe, Inc., San Diego, CA), a DNA probe hybridization test based on the rRNA gene sequence, was used to confirm the identity of all NT isolates (4, 9, 13). When the AccuProbe® test generated equivocal results, two additional runs with the AccuProbe® test were performed, and the result generated two out of three times was considered the final outcome for those isolates.

Identification of *lytA* and *psaA*

A real-time PCR assay was used to identify the presence of the *lytA* and *psaA* genes in a subset of NTs, as described in Carvalho, 2007 (8). All real-time PCR work was performed at the Centers for Disease Control and Prevention in Atlanta, Georgia.

Identification of the capsular locus (*cps)*

One NT isolate was randomly selected per colonized individual, and the presence of the capsular locus (*cps*) was determined with PCR primers directed to the conserved *wzg* (*cpsA*) gene: Wzg-f: 5'ATCCTTGTCAGCTCTGTGTC3' and Wzg-r: 5'TCACTTGCAACTACATGAAC3' (18). DNA extracts generated from the multiplex PCR serotyping protocol were thawed at room temperature and used in the *wzg* PCR protocol. The PCR protocol used in this study is as follows: 94°C for 4.0 minutes; 94°C

for 45 seconds, 55°C for 45 seconds, 72°C for 2.0 minutes (x 29); 72°C for 6.0 minutes, 4°C hold. Each PCR reaction contained 100 pmol/µl of each forward and reverse primer, 10mM of dNTPs (New England BioLabs), 25mM $MgCl^{2+}$, and GoTaq® Flexi DNA Polymerase (Promega) with associated 5x buffer in 50µl reactions. Every PCR run included *Streptococcus pneumoniae* ATCC® 49619 (serotype 19F) as a positive control and a no-template (dH_2O) negative control. PCR products were electrophoresed on 1% agarose gels in TAE buffer at 120 V for approximately 40 minutes. Gels were stained in ethidium bromide (0.5 µg/mL), and PCR product sizes were compared to a 50-bp molecular standard (Novagen, Inc). A positive result for *wzg* was indicated by amplification of a single 1,800 bp product.

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) for penicillin, cefotaxime, ceftriaxone, chloramphenicol, ertapenem, erythromycin, levofloxacin, linezolid, moxifloxacin, ofloxacin, telithromycin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin were determined using the AST-GP65 card for the Vitek® 2 system (bioMerieux, France) (1, 11, 17, 23). For susceptibility testing, frozen pneumococcal isolates were subcultured twice on TSA II and incubated for 20-24 hours at 37°C in 5% CO_2. *S. pneumoniae* ATCC® 49619 served as the quality control and was grown in ambient air, in accordance with Vitek® 2 system recommendations. MIC cut-points were defined according to the CLSI criteria (2007) (12). Multidrug resistance (MDR) was defined as resistance to three or more different classes of antibiotics.

Selection of unique pneumococcal strains

It was presumed that the multiple colonies isolated per colonized individual would most likely be the same strain. To test this assumption, we compared the serotypes and antibiograms of each pneumococcal isolate to the other co-colonizing isolates from the same individual. Co-colonizing pneumococci from the same individual were considered different if they had different serotypes (or, a typeable vs. nontypeable isolate) or the same type but different antibiograms. Antibiograms were considered different if the MIC value of at least one antibiotic differed from the other pneumococci colonizing the same individual. MIC values were considered different for antibiotics if they diverged by more than one dilution factor above or below the MIC value. One isolate was randomly selected for further analysis from each group of identical pneumococci colonizing an individual.

Statistical analyses

All statistical analyses were performed with SAS v 9.1.3 (SAS Institute, Cary, NC). Logistic regression was used to compute chi-square statistics, odds ratios (ORs), and 95% confidence intervals to investigate the frequency of carrying a nontypeable pneumococcus by age group. From cross tabulations, the likelihood of antibiotic nonsusceptibility among nontypeable vs. typeable pneumococci was assessed using chi-square statistics, and the magnitude of the association was estimated by ORs and 95% confidence intervals.

Results

Identification of nontypeable (NT) pneumococci

A total of 568 pneumococci were isolated from 56 colonized individuals; 29.6% of all isolates (n=168) were nontypeable. The 168 NT pneumococci were isolated from 20 individuals; of these, 29 unique NT strains were selected on the basis of their antibiograms (having the MIC of at least one antibiotic differ from the other co-colonizing pneumococci) for analysis. A total of 400 encapsulated pneumococci were also isolated from 39 individuals in the study, and 67 unique encapsulated strains were selected on the basis of their antibiograms for analytic comparison with the NTs (i.e., 96 unique pneumococcal isolates (67 typeable and 29 NT) were obtained from 56 individuals and were used for analysis in this study).

All NTs were optochin sensitive, bile soluble, failed to react with traditional antisera or gave a negative result with a PCR-based method, and were AccuProbe® pneumococcus culture test positive. We did not detect the presence of any atypical (i.e., optochin resistant or bile insoluble) pneumococci in our collection of NTs.

To ensure the NT isolates were true pneumococci and not closely related viridans streptococci, a subset of isolates was tested for the presence of the pneumococcal specific *lytA* and *psaA* genes. All tested NT pneumococci were positive for the pneumococcal surface adhesion A (*psaA*) gene, while only two isolates were positive for the gene that encodes the major autolysin (*lytA*) (Table 4.1). A different subset of NTs was tested for the presence of the conserved capsular gene *wzg* (*cpsA*). All tested NTs appeared to lack the *cps* locus (Figure 4.1).

Proportion of colonized individuals carrying NT pneumococci by age group

The proportion of colonized individuals carrying NT pneumococci varied by age group. Among colonized individuals, the highest percentage of NT carriage occurred in the 11-17 and ≥ 50 year age groups (Table 4.2). Thirty-three percent of colonized children ≤ 5 years and 50% of colonized older children and adolescents (6-17 years) carried NT pneumococci. Forty-two percent of colonized adults (≥ 18 years) carried NT pneumococci.

Examining all colonized individuals together, age appeared to be positively associated with carrying a NT pneumococcus, although this relationship was not statistically significant. Colonized individuals who were six years and older may be more likely than colonized children ≤ 5 years to carry a NT pneumococcus (OR: 1.32, 95% CI: 0.49-3.58).

Antibiotic resistance among NT pneumococci

Among the 14 tested antibiotics, NT pneumococci (n=29) exhibited intermediate resistance to the following six drugs: penicillin (37.9%), cefotaxime (17.2%), ceftriaxone (3.5%), erythromycin (6.9%), tetracycline (6.9%), and trimethoprim-sulfamethoxazole (10.3%). NT strains demonstrated full resistance to: penicillin (58.6%), ceftriaxone (3.5%), chloramphenicol (20.7%), erythromycin (79.3%), tetracycline (89.7%), and trimethoprim-sulfamethoxazole (86.2%) (Figure 4.2). NT pneumococci were fully susceptible to ertapenem, levofloxacin, linezolid, moxifloxacin, ofloxacin, telithromycin, and vancomycin.

NT pneumococci were more likely than the 67 encapsulated strains to be nonsusceptible to erythromycin and tetracycline (Table 4.3). There was no difference in

the likelihood of nonsusceptibility to penicillin, cefotaxime, chloramphenicol, or trimethoprim-sulfamethoxazole among NT and typeable isolates. Eighty-three percent of NT pneumococci were multidrug resistant (MDR, resistant to three or more classes of antibiotics), while 58% of encapsulated strains were MDR. NT isolates were 3.4 times more likely than encapsulated strains to be MDR (95% CI: 1.2, 10.1).

Fifty-two percent of NTs exhibited the same antibiogram, hallmarked by nonsusceptibility to penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole. The second most common antibiogram, exhibited by 13.8% of the NTs, was characterized by nonsusceptibility to penicillin, chloramphenicol, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole. The third most common antibiogram (10.3% of NTs) was characterized by nonsusceptibility to penicillin, tetracycline, and trimethoprim-sulfamethoxazole.

Discussion

We discovered an unusually high percent of NT pneumococci (30%) among colonized household members of all ages in Nha Trang, Vietnam. The proportion of colonization with NT pneumococci appeared to increase with age, and 83% of NT isolates were resistant to three or more classes of antibiotics.

Parry, et al (2000), reported that 6.4% of 125 pneumococcal isolates carried by 404 children in Vietnam were nontypeable (33). Another study of pneumococcal carriage conducted in 2007 among 1,422 children in Vietnam indicated that 19.7% of 178 isolates were NT (39). The highest percent of colonizing pneumococci that were NT was reported by López, et al, in a 1999 study of pneumococcal carriage among 332 six year-

old children in northern Spain (24). The authors showed that 44.5% of 128 isolated pneumococcal strains were NT, and these strains were frequently multidrug resistant (30%). In the only other carriage study that included children and adults, 5% of colonized Israeli children ≤ 6 years (n=404) and 24% of colonized adults ≥ 18 years (n=1,300) carried NT pneumococci (37). By contrast, in Nha Trang, Vietnam, 33% of colonized children ≤ 5 years, 50% of colonized 6-17 year-olds, and 38% of colonized adults ≥ 18 years carried NT strains.

There are at least three explanations for the overall high percentage of NT strains captured here. First, there may be true differences in the type of pneumococcal strains carried by young children versus older children and adults that have previously gone unrecognized. The preponderance of pneumococcal colonization studies only enroll children ≤ 5 years, as this age group has the highest burden of pneumococcal disease, is primarily responsible for maintaining endemic transmission, and is targeted by recently-licensed serotype-specific conjugate vaccines. If other age groups are enrolled in colonization surveys, it is usually limited to the parents or primary caregivers of a young child. By examining healthy individuals of all ages, we may have captured a more accurate reflection of the typical carried pneumococcal population. Thus, the average pneumococcal population may be composed of a greater proportion of NT strains than previously acknowledged.

Second, our high percent of NT pneumococci may be related to the local epidemiology in Nha Trang, Vietnam, which may not be indicative of transmission of NT pneumococci elsewhere. We cannot exclude the possibility that an undetected outbreak of conjunctivitis due to a NT pneumococcus may have occurred prior or during the

current study. Additionally, it is possible that a particularly successful strain or strains of NT pneumococci has recently invaded Vietnam. Parry, et al., reported in 2000 that 6% of pneumococcal isolates carried by colonized children in Vietnam were NT, while Schultsz and colleagues reported that almost 20% of pneumococci carried by young children in Vietnam in 2003-2004 were NT (33, 39). We observed in 2006 that 33% of carried pneumococci among children five years and younger were NT. Comparisons between these three studies should be made cautiously, given the different geographic locations and sampling procedures (nasal vs. nasopharyngeal swabs), but a particularly successful strain of NT pneumococcus that has recently established itself and been able to persist in Vietnam may also explain our ascertainment of such a high percentage of NT pneumococci.

Third, our inclusive selection of up to 10 pneumococcal isolates per individual may have allowed us to capture both encapsulated pneumococci, with their characteristic large, wet colony morphology that is usually selected by laboratorians, and nontypeable pneumococci, which are smaller and dry in appearance and not always chosen if only one colony per person is the guiding selection protocol. Thus, our laboratory methods may have enabled us to describe a relatively ignored aspect of the typical carried pneumococcal population.

In the current study, pneumococcal colonization declined overall with age, but the fraction of carried pneumococci that were NT increased with age. While the sample size of our older age groups was small, this observation may still have implications for understanding the immune response to pneumococci during carriage and for future sampling strategies of adolescents and adults. Serotype-specific anti-capsular antibodies,

as a result of serial colonization, have long been assumed to be the basis of immunity to pneumococci. However, some investigators have questioned this assumption, since carriage and disease caused by all encapsulated pneumococci declines with age, not just carriage and disease caused by the limited number of serotypes to which individuals have acquired immunity. These investigators have argued that antibody-based immunity may be due to some common component of the pneumococcal capsule or other common antigen. No investigator has previously reported an increase in the fraction of carriage due to NT pneumococci with increasing age, which may suggest that antibody-based immunity is to some common component of the pneumococcal capsule. Alternatively, immunity may be to some non-capsule-specific component that is present among encapsulated strains, but not all NT pneumococci. Such immunity would result in a decline in the fraction of carriage due to encapsulated strains with age, but allow for an increase in the fraction of carriage due to NT strains.

The NT pneumococci isolated in this study were highly drug resistant and were three times more likely than encapsulated strains from the same sampled population to be MDR. Porat, et al. (2006), reported a lower proportion of resistance among NT pneumococci isolated in Israel compared to the present study. The authors examined 148 nontypeable pneumococci isolated from the nasopharynges, middle ear fluid, and conjunctiva of 404 children < 3 years in Israel and observed that 74% were penicillin nonsusceptible, 49% of the isolates were resistant to trimethoprim-sulfamethoxazole, 29% were resistant to tetracycline, 25% were resistant to erythromycin, and 30% of isolates were resistant to ≥ 3 drugs. The greatest percentage of resistance was found among nasopharyngeal isolates. When the authors compared the proportion of resistance

among the 148 nontypeable isolates to the additional 5,352 typeable pneumococci also recovered in their study (from carriage samples, middle ear fluids, conjunctiva, and blood and cerebrospinal fluid cultures), antimicrobial nonsusceptibility percentages in their entire pneumococcal sample were comparable to those of the nontypeable isolates. The fact that the authors combined samples from five anatomic sites in their analysis, but showed that the frequency of resistance was highest among nasopharyngeal isolates, most likely accounts for the differences in resistance frequency between Porat's study and the current investigation (which only examined nasopharyngeal isolates).

The observation that NT pneumococci are more likely to be MDR than encapsulated colonizing strains may be related to their genetic plasticity owing to a lack of capsular expression, their ability to form biofilms, or the length of time some NTs have been circulating among the human population, being exposed to antibiotics (3, 29, 38, 41). The prevalence of drug resistance among NTs is cause for concern, since NT strains also contribute to the pool of resistance determinants available to the encapsulated pneumococcal population (19, 43). For example, Hauser, et al., reported that some isolates of the internationally-spread *S. pneumoniae* serotype 19F clone that emerged in Switzerland had evolved from low-level penicillin resistance to higher resistance levels, most likely via acquisition of penicillin binding protein (pbp2x) gene fragments from local nonencapsulated pneumococci (20). It is also theoretically possible that NT pneumococci may acquire or re-acquire the genes for capsular expression, resulting in bacteria that are highly drug-resistant and capable of causing invasive disease (5).

In the current study, we defined NT pneumococci according to the classic phenotypic definition, with the addition of the AccuProbe® pneumococcus test criterion.

That is, bacterial isolates were defined as NT pneumococci if they formed α-hemolytic colonies on blood agar, were optochin susceptible when grown in 5% CO_2, bile soluble, failed to react to antipneumococcal capsular antibodies, and were AccuProbe® pneumococcus test positive. While non-pneumococcal oral streptococci may exhibit colony morphologies that are very similar to pneumococci, they are classically optochin resistant, bile insoluble, and do not react with antipolysaccharide antibodies. We also investigated whether our NT pneumococci lacked capsular genes versus failed to express a capsule. Testing of a subset of our NT isolates indicated that they are nonencapsulated pneumococci that have lost the genes for capsular production, rather than having disrupted capsular expression (18). Thus, based on the currently accepted definition, we consider these isolates to be true NT pneumococci that appear to have lost the biosynthetic capsular locus.

In an attempt to further characterize these isolates, we tested a subset of our strains for the presence of the *lytA* gene (encodes for a choline-binding protein and is the major autolysin) and the *psaA* gene (encodes for the pneumococcal surface adhesion A, an adhesion expressed on the surface of all pneumococci). Much investigation has gone into describing the *lytA* and *psaA* genes and their associated protein molecules, as these gene targets serve as the basis of many molecular diagnostic approaches for pneumococci. LytA and PsaA are also two main antigen candidates for protein-based pneumococcal vaccines currently under development. A recent study using clinical isolates showed that true pneumococci will be positive for both the *lytA* and *psaA* genes, while pneumococcus-like viridans group streptococci will lack both genes, and *S. pseudopneumoniae* (the closest evolutionary relative to *S. pneumoniae*) may have either

or both of the genes (8). All of our tested isolates were positive for *psaA*, but only 18% of NTs were positive for *lytA*. However, since our NT isolates were all optochin susceptible when grown in 5% CO_2, and *S. pseudopneumoniae* is optochin resistant when grown under these conditions, we contend these isolates are not *S. pseudopneumoniae*. In addition, all of our NTs were also soluble in deoxycholate (bile salts), while *S. pseudopneumoniae* is insoluble in bile salts. Since deoxycholate activates LytA and results in the bile soluble phenotype, our strains are phenotypically LytA positive. It is important to note that the real-time PCR primers used to detect *lytA* are pneumococcal specific; a negative result does not necessarily indicate that these isolates do not have a *lytA* gene or make a LytA protein. Our NT isolates may exhibit a variant of *lytA* that is not detectable by the real-time PCR primers that were developed on the basis of clinical pneumococcal isolates. Alternatively, our NT pneumococci may have some altered autolytic system that may affect the pathogenic potential of these strains. The *lytA* gene of NT pneumococci may show more diversity than encapsulated strains, where the *lytA* gene has been demonstrated to display restricted allelic variation despite localized recombination events with genes of pneumococcal bacteriophage encoding cell wall lytic enzymes, presumably because of the importance of LytA in the pathogenesis of encapsulated pneumococci (42). NTs may exhibit a greater variation in their *lytA* genes that have lead to LytA-like amidases that are less pathogenic. Further investigation into the allelic diversity of *lytA* genes from NT pneumococci is warranted to substantiate this theory.

Although it is improbable that these nonencapsulated isolates are *S. pseudopneumoniae* on the basis of the phenotypic reactions, the observation that 82% of

the tested isolates potentially lack the *lytA* gene warrants further comment. After a careful review of the literature, we could find no other report of pneumococci or other viridians streptococci that matched the phenotypic and genotypic results documented here. Assuming that the real-time PCR *lytA* and *psaA* results are correct, it is possible that these isolates represent a previously unrecognized streptococcal species that are evolutionarily in between *S. pneumoniae* and its closest evolutionary relatives (*S. pseudopneumoniae*, *S. mitis*, and *S. oralis*). In 2008, Kilian and coworkers combined population genetic analysis of 118 α-hemolytic streptococci (tentatively assigned to the species *S. pneumoniae*, *S. mitis*, *S. oralis*, *S. infantis*, and *S. pseudopneumoniae*) with categorization by a distinct cell wall carbohydrate structure and competence pheromone sequence signature to show that *S. pneumoniae* is one of several hundred evolutionary lineages forming a cluster separate from *S. oralis* and *S. infantis* (21). Based on the evolutionary age of the lineages, the identical location of remnants of virulence genes in the genomes of commensal strains, the pattern of genome reductions, and the proportion of unique genes and their origin, the authors propose that the entire cluster of *S. pneumoniae*, *S. pseudopneumoniae*, and *S. mitis* lineages evolved from pneumococcus-like bacteria with all the properties associated with virulence and, presumably, pathogenic to the common immediate ancestor of hominoids. It was during adaptation to a commensal lifestyle that most of the lineages lost the majority of genes determining virulence and became genetically distinct due to sexual selection in their respective hosts (21). The results of Kilian, et al., suggest that commensal streptococci gradually evolved from a pathogen by genome reduction. The authors also hypothesize that, while the pneumococcal lineage conserved expression of both capsule production and IgA1

protease activity to ensure their ability to colonize in the presence of IgA1 antibodies, lineages evolving into a commensal lifestyle gradually lost both characteristics and achieved the colonization advantage of the capsule-deficient phenotype (21). Kilian, et al., propose that the isolation of capsule-deficient pneumococci may be an indication of the same process occurring within the pneumococcal lineage, but at a slower rate (21). Thus, our isolation of capsule-deficient pneumococci, the majority of which potentially lack the gene encoding for the major autolysin (*lytA*), may reveal an ongoing evolutionary process toward decreased virulence and enhanced colonization ability among a subset of the pneumococcal population.

Since the vast majority of previous investigators classified NT pneumococci as α-hemolytic bacterial isolates that are optochin susceptible, bile soluble, and failed to react with antipneumococcal capsule antisera, with no description of associated virulence genes, it is difficult to determine if our *lytA* observation is similar to that among other NT pneumococci. Whatmore, et al., reported the presence of *lytA* and *ply* in 15 of 16 nontypeable pneumococci isolated from disease contexts, and three nontypeable pneumococci isolated from conjunctivitis outbreaks in the United States were positive for *lytA*, *ply*, and *psaA* (8, 43). It is unknown whether NT pneumococci isolated from healthy carriers (as in our study) would be more likely to lack *lytA* and other virulence genes, as compared to disease-causing NT pneumococci. Until the necessary investigation is performed to resolve these issues of identification and pathogenic potential of these strains, we base our definition of NT pneumococci on the currently accepted phenotypic criteria, while acknowledging the deficits of this approach.

It is interesting to speculate on what factors or characteristics make these NT pneumococci particularly successful in Vietnam and, more broadly, how NT pneumococci persist (in general) when the pneumococcal population is dominated by encapsulated, more virulent strains. The high level of antibiotic resistance exhibited by the NTs in Vietnam may provide a key insight. Our NTs were over three times more likely to be multidrug resistant than the encapsulated pneumococci, which would be highly advantageous in Vietnam, where overuse of antibiotics is rampant. The most common antibiogram exhibited by 52% of the NTs and 40% of the encapsulated pneumococci in this study featured nonsusceptibility to penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole, reflecting the antibiotics most frequently used in Vietnam. While both sub-groups of bacteria were commonly resistant to the same antibiotics, the NTs more commonly showed this multidrug resistance profile and may (in part) explain the success of these strains in Vietnam.

There also may be universal features of NT pneumococci that, coupled with a high level of resistance due to the selective pressure of antibiotics in Vietnam, favor the success of these nonencapsulated, drug resistant strains. The NT pneumococci isolated in this study appeared to lack the genes for capsular biosynthesis. These strains may have adopted a different 'lifestyle' than their encapsulated counterparts to thrive in an ecological niche dominated by more virulent strains. For example, in order to evade the host immune response, NT pneumococci may vary their surface structures more frequently than encapsulated strains, similar to nontypeable *Haemophilus influenzae*. NT pneumococci may also form biofilms more readily than their encapsulated counterparts, as another strategy to evade the host immune response. Enhanced biofilm-forming

ability would also correspond to the greater prevalence of antibiotic resistance observed among the NT pneumococci in the present study. NT pneumococci may also colonize the nasopharynx for a longer period of time, compared to encapsulated pneumococci. Unencapsulated pneumococci have been shown to have increased adherence to respiratory epithelial cells when compared to encapsulated strains, which may allow NTs to persist in the nasopharynx for a greater duration (25). Colonizing the nasopharynx for a longer time period would also increase the likelihood of encountering the selective pressure of antibiotics, and could also factor into the greater drug resistance observed among these isolates.

Finally, if transmission to a new host is the ultimate 'goal' of a microbe, NT pneumococci may have evolved to be less virulent but highly transmissible organisms in order to be maintained in a population. NTs have been associated with large, explosive outbreaks of conjunctivitis, as well as sporadic cases of the illness. It is interesting to note that NT pneumococci appear to have a particular tropism for conjunctiva; access to the conjunctiva enable the bacterium to cause disease symptoms, increasing the probably of being transmitted to a new host. A longer duration of colonization would also increase the transmission possibilities for NT pneumococci by providing more ongoing, lower-level transmission, as opposed to the outbreak scenario.

In conclusion, we observed an unusually high percent of NT pneumococci among colonized individuals of all ages in Vietnam. The NT pneumococci were highly drug resistant, and the fraction of colonization with NTs appeared to increase with age. Although study of NT pneumococci has been largely ignored and plagued by identification issues, these strains appear to be an important part of the recombining

pneumococcal population that have persisted among humans for years, have the ability to cause disease, and can contribute to the pool of resistance determinants accessible to all pneumococci. While much future work is necessary to establish the pathogenic potential and exact phylogenetic position of NTs among the *S. pneumoniae-pseudopneumoniae-mitis* cluster, the existence of this segment of the pneumococcal population should be considered in sampling strategies for future carriage studies and vaccine design approaches. Our unexpected observation of approximately half of colonized adolescents and adults carrying NTs highlights the necessity of sampling all age groups in colonization studies. Furthermore, the introduction of PCVs may result in an increased prevalence of NTs; this consequence could be temporarily beneficial, if the selective pressure due to a PCV results in a greater proportion of unencapsulated, potentially less virulent pneumococci. However, this benefit may be short-lived, considering the high degree of drug resistance among NTs, the theoretical potential of NTs acquiring the genes for capsule production, and the naturally competent characteristic of these bacteria.

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Figure 4.1: Agarose gel from *wzg* PCR to identify *cps* locus in nontypeable (NT) pneumococci.

Lanes 1 and 24 contain 50-bp ladder; lanes 2-21 contain 20 NT pneumococci isolated from nasopharyngeal swabs of 519 healthy household members in Nha Trang, Vietnam (2006); lane 22 contains the negative control; and lane 23 contains the positive control (serotype 19F pneumococcus). Presence of the conserved capsular gene *wzg* (*cpsA*) is indicated by amplification of 1,800 bp product. All of the NT pneumococci are negative for *wzg*, and only the positive control yielded the 1.8 kb product.

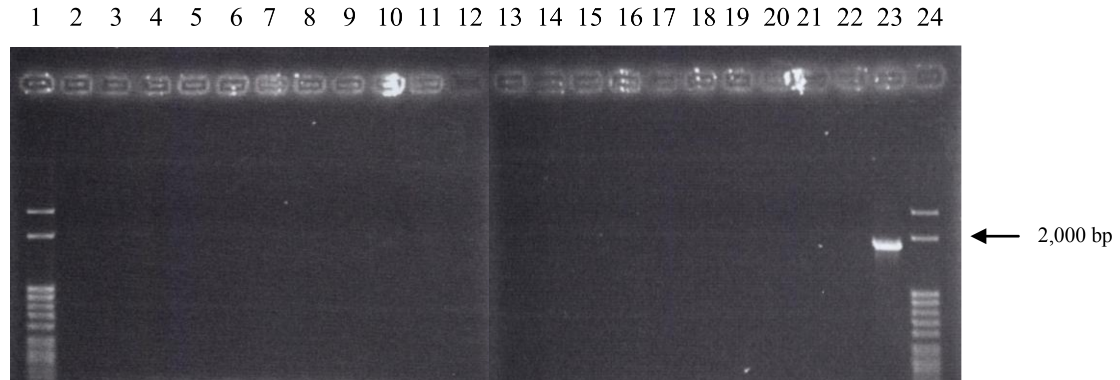
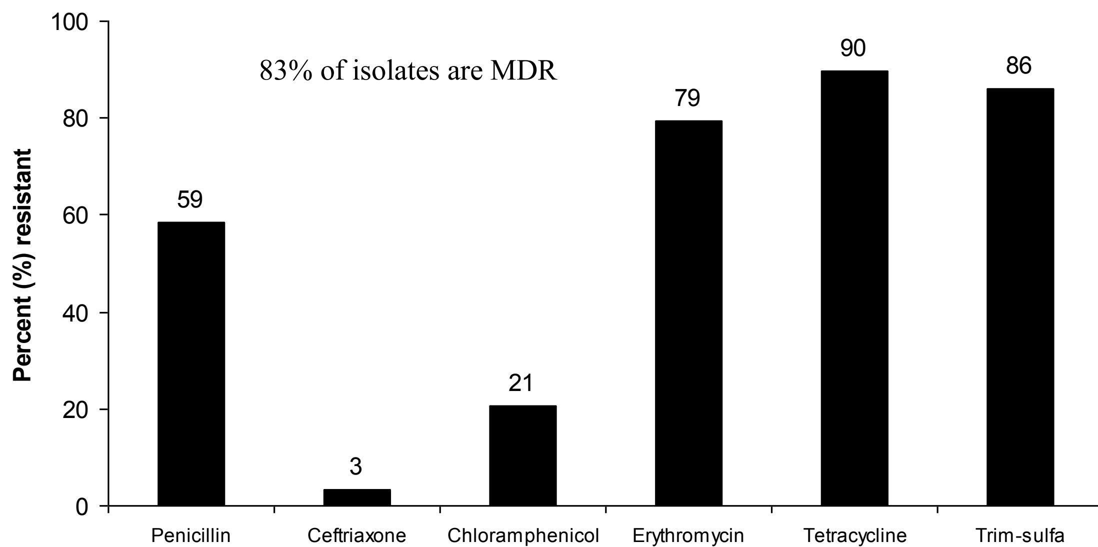


Figure 4.2: Percent of nontypeable (NT) pneumococci that are fully resistant to selected antibiotics (n=29 unique[†] isolates).

NT pneumococci were isolated from nasopharyngeal swabs of 519 healthy household members in Nha Trang, Vietnam, in 2006. All isolates were tested for susceptibility to 14 antibiotics using the AST-GP65 card for the Vitek® 2 system. The percentage of NT pneumococci exhibiting full resistance and multidrug resistance (MDR, resistant to ≥ 3 drugs) are shown below. NT pneumococci were fully susceptible to ertapenem, levofloxacin, linezolid, moxifloxacin, ofloxacin, telithromycin, and vancomycin; 17% of isolates demonstrated only intermediate resistance to cefotaxime.



[†]Co-colonizing pneumococci from the same individual were considered different if they had different serotypes (or, a typeable vs. nontypeable isolate) or the same type but different antibiograms. Antibiograms were considered different if the MIC value of at least one antibiotic differed from the other pneumococci colonizing the same individual. MIC values were considered different for antibiotics if they diverged by more than one dilution factor above or below the MIC value.

Table 4.1: Phenotypic and genotypic identification tests of a subset of NT pneumococci (n=11) isolated from 519 healthy household members in Nha Trang, Vietnam (2006).

All tested NT pneumococci are optochin sensitive, bile soluble, Gen-Probe® AccuProbe® pneumococcus test positive, nontypeable by the Quellung method and multiplex PCR, and have the *psaA* gene. Only two of the 11 tested isolates are positive for the *lytA* gene.

Isolate	Optochin†	Bile Solubility	Gen-Probe® AccuProbe® Pneumococcus test	Serotyping-Quellung‡	Serotyping-Multiplex PCR	Real-time PCR *lytA*	Real-time PCR *psaA*
1430173-11.8	S	+	+	NT	–	–	+
1430305-07.1	S	+	+	NT	–	–	+
1430348-03.1	S	+	+	NT	–	+	+
1432022-04.1	S	+	+	NT	–	–	+
1432048-04.1	S	+	+	NT	–	–	+
1432076-05.9	S	+	+	NT	–	–	+
1432133-03.1	S	+	+	NT	–	–	+
1430387-01.1	S	+	+	NT	–	+	+
1432123-03.1	S	+	+	NT	–	–	+
1432115-03.11	S	+	+	NT	–	–	+
1430217-03.1	S	+	+	NT	–	–	+

† S = Susceptible
‡ NT = Nontypeable

Table 4.2: Carriage of nontypeable (NT) pneumococci by age group.

Fifty-six of 519 healthy, community dwelling individuals in Nha Trang, Vietnam, were colonized with *S. pneumoniae* in October, 2006. Of the 56 colonized individuals, 20 individuals carried NT pneumococci, and the proportion of colonized individuals carrying NT pneumococci may increase with age.

Age (years)	N	No. (%) of individuals colonized	No. of colonized individuals carrying a NT pneumococcus	Percent (%) of colonized individuals carrying a NT pneumococcus	Percent (%) of all individuals carrying a NT pneumococcus
0−2	60	21 (35)	4	19	7
3−5	44	21 (48)	10	48	23
6−10	22	3 (14)	1	33	5
11−17	33	3 (9)	2	67	6
18−30	142	2 (1)	0	0	0
31−49	120	4 (3)	1	25	1
≥ 50	98	2 (2)	2	100	2
Total:	519	56 (11)	20	36	4

Table 4.3: Distribution of nonsusceptibility to nine antibiotics among 29 unique[†] nontypeable (NT) and 67 unique typeable pneumococci isolated from 519 healthy individuals in Nha Trang, Vietnam (October, 2006). All isolates were fully susceptible to ertapenem, levofloxacin, linezolid, moxifloxacin, and vancomycin.

Antibiotic	Frequency (%) of NT pneumococci nonsusceptible to antibiotic (n=29)	Frequency (%) of typeable pneumococci nonsusceptible to antibiotic (n=67)	OR (95% CI)
Penicillin	28 (97)	62 (93)	2.26 (0.25, 20.24)
Cefotaxime	5 (17)	3 (4)	4.44 (0.99, 20.04)
Ceftriaxone	2 (7)	0 (0)	--
Chloramphenicol	6 (21)	9 (13)	1.68 (0.54, 5.26)
Erythromycin	25 (86)	40 (60)	**4.22 (1.32, 13.50)**
Ofloxacin	0 (0)	2 (3)	--
Telithromycin	0 (0)	1 (1)	--
Tetracycline	28 (97)	51 (76)	**8.78 (1.11, 69.77)**
Trimethoprim-sulfamethoxazole	28 (97)	64 (96)	1.31 (0.13, 13.17)

[†]Co-colonizing pneumococci from the same individual were considered different if they had different serotypes (or, a typeable vs. nontypeable isolate) or the same type but different antibiograms. Antibiograms were considered different if the MIC value of at least one antibiotic differed from the other pneumococci colonizing the same individual. MIC values were considered different for antibiotics if they diverged by more than one dilution factor above or below the MIC value.

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Chapter 5

Applying Sequential Multiplex PCR Capsular Serotyping to Pneumococcal Nasopharyngeal Isolates from Healthy Household Members in Vietnam

Abstract

Background: Monitoring *Streptococcus pneumoniae* serotype distributions among colonizing and invasive isolates is essential for evaluating the impact of conjugate vaccines on transmitted serotypes. However, traditional methods for serotyping *S. pneumoniae* are time-consuming, expensive, and subjective. The recently developed sequential multiplex PCR assay for serotyping invasive disease isolates is highly valuable, but its performance among nasopharyngeal (NP) isolates is unknown. Here, we report our experience applying this multiplex PCR assay to a collection of NP isolates obtained from healthy children and adults in Vietnam.

Methods: The Latin American (LA) formulation of the sequential multiplex PCR was used to serotype 568 NP isolates from healthy children and adults living in Nha Trang, Vietnam. Any isolate that could not be typed with either the multiplex PCR or antisera was considered to be nontypeable (NT). The identity of all NT isolates was confirmed by optochin disk and bile solubility testing and the Gen-Probe® AccuProbe® system.

Results: Fifty-four percent of all pneumococci were serotyped in PCR reaction one, and 70% were typed by reaction six. NT pneumococci appeared as blank (*cpsA* negative) or generated a multiple primer banding pattern on electrophoresis gels.

Among just encapsulated pneumococci, the multiplex PCR serotyped 76% of NP isolates in reaction one and 100% of isolates by reaction six.

Discussion: This sequential multiplex PCR assay is an efficient initial approach for typing NP isolates. Traditional typing methods will have to be employed for NT pneumococci and any serotype not covered by the assay. The LA formulation of the sequential multiplex PCR does an excellent job of covering the serotypes found in Vietnam and should be considered for use in other countries with similar serotype distributions.

Introduction

Streptococcus pneumoniae (the pneumococcus) is a leading cause of bacterial infection worldwide and causes a range of illness from otitis media and pneumonia to invasive disease, such as meningitis and bacteremia. Globally, *S. pneumoniae* causes up to 1 million deaths in children under 5 years, annually. The greatest mortality due to invasive pneumococcal disease (IPD) is in resource-poor countries, where pneumococcal disease and treatment are complicated by antibiotic resistance, HIV co-infection, and inadequate/inaccessible medical care (9, 11). The human nasopharynx is the natural reservoir of the pneumococcus; asymptomatic colonization is common in young age groups and serves as the main mode of transmission.

There are at least 91 known serotypes of *S. pneumoniae*, based on the structure of the polysaccharide capsule, which prevents opsonophagocytosis and is considered a main virulence factor (22). Introduction of the seven valent pneumococcal conjugate vaccine (PCV7) into pediatric populations worldwide resulted in dramatic declines in IPD due to

vaccine serotypes (VTs, serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) and an approximate 50-65% reduction in carriage of VTs among vacinees (4, 5, 12, 17, 29). Due to its impact on carriage, PCV7 also appears to cause a significant herd effect, resulting in a pronounced reduction in IPD among non-vacinees (24, 29, 30). Post-PCV7 increases in carriage and disease due to non-vaccine serotypes (NVTs) warrant continued monitoring of colonizing and disease-causing pneumococcal serotypes to evaluate the impact of multivalent conjugate vaccines on transmitted serotypes and inform future vaccine composition (4-6, 12, 17, 20, 28, 31).

Traditional antisera-based methods for serotyping pneumococci are time-consuming, expensive, and dependent on laboratorian skill and experience. A recently developed sequential multiplex PCR assay for serotyping invasive disease isolates is highly accurate and efficient, but its performance with NP isolates has not been demonstrated (8, 18, 21). There are also three different formulations of the multiplex PCR, based on the serotype distributions found in the United States, Africa, and Latin America (LA). The PCR scheme that could most efficiently type isolates from Asia, which has a high burden of IPD and drug-resistant pneumococci, is uncertain.

Here, we demonstrate that the LA formulation of the multiplex PCR assay is highly accurate for serotyping NP isolates by applying it to a collection of carriage isolates from healthy children and adults in Vietnam. The LA formulation was selected because it includes serotypes 14, 6A/B, 19F, and 23F in its first reaction, and these serotypes were shown in a limited number of studies to be commonly carried by children in Vietnam (15, 23, 26, 27). The US or African schemes would yield the same serotypes as the LA formulation, but with less efficiency, because of the different serotype primer

combinations in each multiplex reaction (i.e., an increased number of PCR reactions per isolate would be necessary to obtain the same result). We show that the LA scheme is extremely efficient for serotyping carriage isolates from a country in Southeast Asia and recommend it for use in other countries where serotypes 14, 6A/B, 19F, and 23F dominate.

Methods

Bacterial isolates and culture conditions

A cross-sectional study of pneumococcal carriage was conducted among 115 households in one hamlet of Vinh Thanh commune, Nha Trang, Vietnam, during October, 2006. A single calcium alginate nasopharyngeal (NP) swab (Calgiswab® Type 1, Puritan Medical Products Company LLC, Guilford, ME) was obtained from 519 healthy, community-dwelling children (n=159) and adults (n=360) of all ages. NP swabs were immediately inoculated into STGG transport media, stored at -20°C for one week, shipped on dry ice to the University of Michigan, and stored at -80°C (19). All study procedures were approved by the Health Sciences Institutional Review Board (IRB) at the University of Michigan (HUM00006257), the IRB of the National Institute of Hygiene and Epidemiology in Hanoi, Vietnam, and the IRB of the International Vaccine Institute in Seoul, Korea (2006-009).

Frozen vials of STGG medium containing NP swab specimens were warmed in a 37°C water bath for 20 minutes and then vortexed for 20 seconds. Thawed samples in media (50 µl) were inoculated on trypticase soy agar with 5% sheep blood (TSA II) containing gentamicin (2.5 mg/L) and separately on non-selective media (TSA II). All

specimens were incubated at 37°C in 5% CO_2 overnight (O/N) (19). Presumptive identification of pneumococcal colonies was based on colony morphology and α-hemolysis on blood agar. Up to 10 pneumococcal colonies were sampled per individual. If more than one pneumococcal colony morphology was present, then up to 10 colonies per morphology type were isolated.

Optochin sensitivity, bile solubility, and Gram stain

Identification of pneumococci was based on optochin sensitivity (zone ≥14 mm with 6 mm P discs after O/N incubation at 37°C in 5% CO_2), solubility in 10% deoxycholate using the plate method, and Gram stain (3, 14, 19). Isolates that were bile soluble but exhibited intermediate (6-13 mm zone) or full (no zone) optochin resistance were retained for serotyping, as were bile insoluble but optochin sensitive isolates (1).

DNA extraction and multiplex PCR

DNA was extracted from each pneumococcal isolate, as previously described (21). The Latin American (LA) formulation of the sequential multiplex PCR was used for serotyping (8). The LA scheme consists of six multiplex PCR reactions performed consecutively until a capsular genotype is assigned to each pneumococcal isolate. Any isolate that cannot be typed after the six PCR reactions is subjected to traditional antisera-based serotyping. Isolates that cannot be typed with the sequential multiplex PCR and fail to react with antisera are considered nontypeable. Table 5.1 shows the 30 serotypes targeted by the primers in the six multiplex PCR reactions.

Each PCR reaction included *Streptococcus pneumoniae* ATCC® 49619 (serotype 19F) as a positive control and a no-template (dH_2O) negative control. The positive control yielded two bands where expected in PCR reaction one and one band corresponding to *cpsA* in all subsequent reactions. PCR products were electrophoresed on 2% NuSieve® GTG® agarose gels in TAE buffer at 125-128 V for 38 minutes. Gels were stained in ethidium bromide (0.5 µg/mL), and PCR product sizes were compared to a 50-bp molecular standard (Novagen, Inc).

Conventional serotyping

A 20% random selection of PCR-designated serotypes was confirmed with latex agglutination followed by the Quellung reaction (Statens Serum Institut, Denmark).

Nontypeable pneumococci

Any isolate that was optochin sensitive and bile soluble but could not be typed with either the multiplex PCR or antisera was considered to be nontypeable (NT). The AccuProbe® Gen-Probe® pneumococcus culture identification test (Gen-Probe, Inc., San Diego, CA), a DNA probe hybridization test based on the rRNA gene sequence, was used to confirm the identity of all NT isolates (7, 10).

Results

A total of 568 pneumococci were isolated from 56 colonized individuals. Seventy percent of carriage isolates were encapsulated (n=400) and were typed by the Latin American formulation of the multiplex PCR. The serotype distribution was as follows:

19F (23.0%), 23F (15.8%), 14 (13.5%), 6B (13.0%), 6A (10.8%), 15B/C (11.0%), 11A (8.0%), 16F (2.5%), and 34 (2.5%).

Thirty percent of isolates were determined to be nontypeable (NT) pneumococci (n=168). Eighty-three percent of the NT pneumococci generated a pattern of nonspecific primer banding on electrophoresis gels, while the remaining NTs appeared as blank (negative for the internal positive control, *cpsA*) on gels (Figures 5.1-5.2).

There was 100% correspondence between the PCR results and traditional antisera typing (Table 5.2). All PCR-designated serotypes matched the antisera results, and all isolates that were *cpsA* negative by PCR were nontypeable pneumococci. If encapsulated and nonencapsulated pneumococci are considered together, 54% of all carriage isolates were serotyped in PCR reaction one, and 70% were typed by reaction six. Among just encapsulated pneumococci, the multiplex PCR serotyped 76% of NP isolates in reaction one and 100% of isolates by reaction six.

Discussion

The goals of the present investigation were to determine the ability of a recently developed sequential multiplex PCR assay to type pneumococcal nasopharyngeal (NP) isolates and to demonstrate the efficiency of the Latin American (LA) formulation of the assay for typing isolates from Vietnam. We demonstrated excellent correspondence between the PCR-designated results and traditional serotyping methods for carriage isolates (100% sensitivity and specificity for typeable and nontypeable pneumococci). Considering encapsulated and nonencapsulated pneumococci together, 54% of all carriage isolates were serotyped in PCR reaction one, and 70% were typed by reaction

six. Among just encapsulated pneumococci, the multiplex PCR serotyped 76% of NP isolates in reaction one and 100% of isolates by reaction six.

Thirty percent of all pneumococcal isolates from this carriage study were nontypeable by the multiplex PCR and failed to react with traditional antisera (latex agglutination and Quellung). The majority of prior colonization studies have reported approximately 5-10% of pneumococci from children and up to 24% among adults to be nontypeable (2, 25). One investigation of pneumococcal carriage among 6-year-olds in northern Spain showed that 45% of isolated strains were NT (16). While 30% is among the highest recovery of nontypeable pneumococci from non-sterile site isolates reported to date, these strains were all optochin positive and bile soluble (i.e., 'typical pneumococci') and positive by the Gen-Probe® AccuProbe® pneumococcus test. Based on these criteria, we defined these isolates to be nontypeable (NT) pneumococci. While it is possible that these designated NT pneumococci may be closely related streptococcal species of the mitis group, future DNA-DNA reassociation experiments would be needed to verify their identity (2, 13). Furthermore, we may have recovered a greater than anticipated percentage of NTs due to our selection of up to 10 pneumococcal colonies per person. We selected colonies that exhibited the characteristic α-hemolytic, larger, mucoid morphology and colonies that were α-hemolytic, smaller, and dry in appearance. We may have captured a greater number of NTs using this selection process, compared to other studies where only one or a few characteristic-looking colonies were isolated per person. Finally, this high percentage of NT pneumococci may be due to our inclusion of individuals of all ages in the carriage study. The percentage of individuals colonized with a NT strain was greater for people six years and older, compared to children five

years and younger (see Thesis Chapter 4). Thus, our high recovery of NT pneumococci may reflect strain differences carried by different age groups.

In this study, NT isolates appeared as blank on electrophoresis gels or generated patterns of nonspecific primer binding. Other investigators who have used sterile-site NT isolates in all three of the multiplex PCR formulations (US, Africa, and LA) have observed clear results after running PCR products on gels, but not the nonspecific primer binding that we observed (MDG Carvalho, personal communication). The nonspecific primer binding may be due to the lack of capsular genes among the NTs (see Thesis Chapter 4). Alternatively, we used a higher-grade agarose for electrophoresis (NuSieve® Genetic Technology Grade (GTG)® agarose) than the molecular biology grade agarose (NuSieve® 3:1 agarose) recommended for use with this multiplex PCR assay. We chose the GTG agarose over the non-GTG agarose because the product was available in our lab. The subsequent higher resolution may have enabled observation of the nonspecific primer binding patterns.

The recently developed sequential multiplex PCR assay is a sensitive and accurate method for pneumococcal capsular typing (8, 18, 21). The DNA extraction and PCR protocols are straightforward and inexpensive, requiring only basic laboratory equipment and training, and it is accurate for both clinical and NP isolates. These characteristics will make the assay especially beneficial for resource-poor nations, where antisera for conventional serotyping may be unaffordable, and carriage studies may provide a vital source of serotype distribution information. The sequential multiplex assay currently covers 30 serotypes and is being expanded to include additional serotypes (MDG Carvalho, personal communication), making it a valuable tool to monitor post-vaccine

changes in serotype distributions. In particular, the LA formulation of the sequential multiplex PCR did an excellent job typing NP isolates from Vietnam and should be considered for use in other Asian countries with similar serotype distributions, testing reactions 1, 3, and 4 first. Traditional typing methods will have to be employed for NT isolates and any serotype not presently included in the PCR assay.

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Figure 5.1: Example Latin American reaction 1 with nasopharyngeal isolates from healthy children and adults in Vietnam. PCR products (left-to-right) are: A nontypeable (NT) isolate, another NT, 23F, 14, 23F, *cpsA* positive only, 6A/B, 14, and another serotype 14.

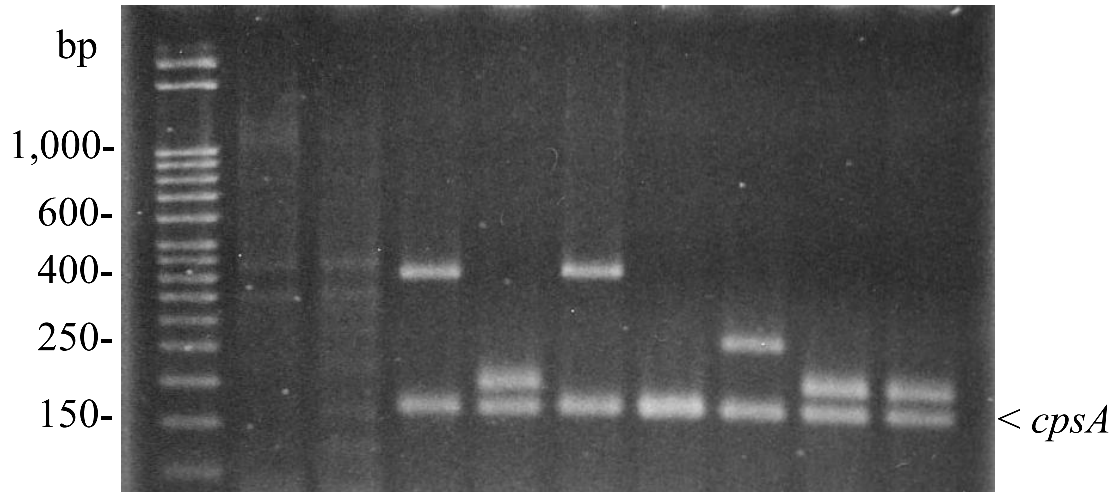

Figure 5.2: Latin American reaction 1 with nontypeable (NT) isolates that are clear on electrophoresis gels. PCR products (left-to-right) are: five NTs, and four serotype 6A/Bs.

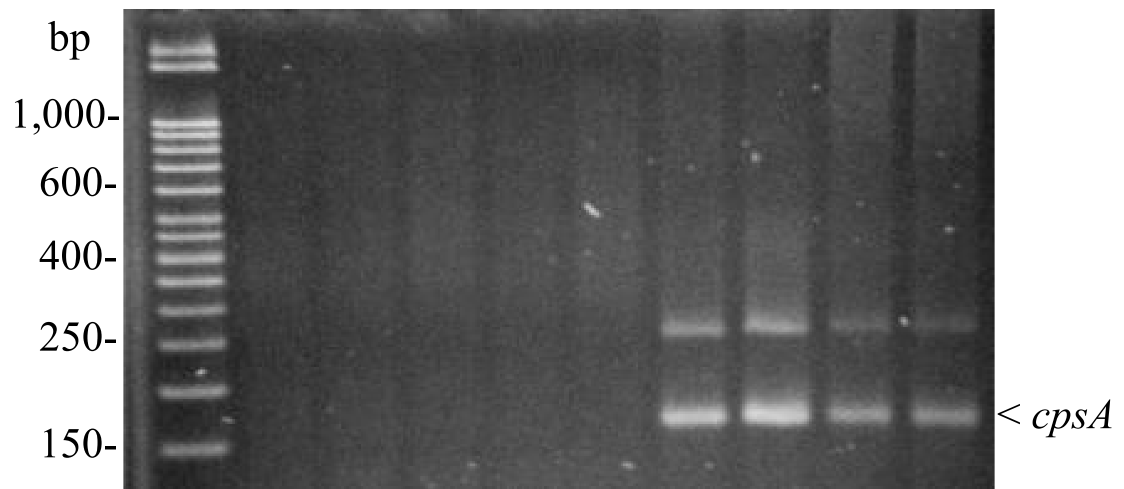

Table 5.1: Thirty pneumococcal serotypes identified by sequential multiplex PCR, using the Latin American formulation. Every pneumococcal isolate is subjected to each multiplex reaction, in order, until assigned a serotype. If no serotype is assigned to an isolate after all six multiplex reactions, then the isolate is typed with traditional antisera.

Multiplex reaction	Primers for Serotypes, per Reaction
1	14, 6A/B, 19F, 23F, 9V
2	1, 5, 4, 19A, 18C
3	3, 11A, 9N/L, 10A, 7F
4	7C, 12F, 15B/C, 38, 17F
5	8, 34, 20, 22F, 31
6	33F, 15A, 35F, 35B, 16F

Table 5.2: Comparison of PCR results and traditional typing methods for 568 pneumococci isolated from a colonization study of healthy children and adults in Nha Trang, Vietnam (2006). Comparison for typeable isolates is based on 20% random selection of strains. All nontypeable isolates were tested with antisera.

Serotype result(s) from PCR	Serotype results using conventional methods (no. of isolates)	Sensitivity (%)	Specificity (%)
19F	19F (15)	100	100
23F	23F (11)	100	100
14	14 (12)	100	100
6A/B/C	6A (11) / 6B (9)	100	100
15B/C	15B (6) / 15C (2)	100	100
11A	11A (6)	100	100
16F	16F (2)	100	100
34	34 (2)	100	100
Nontypeable (clear or nonspecific primer binding on gel)	Nontypeable (168)	100	100

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Chapter 6

Individual and Household-Level Factors Associated with Pneumococcal Colonization: Hypotheses about the Importance of Age-Related Immunity and Contact Patterns to the Epidemiology of *Streptococcus pneumoniae*

Abstract

Background: Individual-level risk factors are not able to fully account for the marked differences in nasopharyngeal (NP) carriage seen across communities. We investigated both individual and group-level factors associated with pneumococcal colonization among healthy individuals of all ages residing in households in one neighborhood in Vietnam prior to pneumococcal conjugate vaccine (PCV) introduction.

Methods: We conducted a cross-sectional study of NP carriage in Khanh Hoa Province, Vietnam, in October 2006. Trained interviewers recruited 519 healthy participants from 115 households in one hamlet of Vinh Thanh commune. Participants were asked demographic, health behavior, and health history questions in face-to-face interviews. One NP swab specimen was collected from each household member. Pneumococci were identified using standard microbiologic procedures, and capsular typing was done with a multiplex PCR approach and conventional serotyping.

Results: Colonization was greatest among children ≤ 5 years and decreased with age. Children ≤ 2 years were more likely to carry PCV serotypes, while older children and adults carried more non-PCV serotypes. Adults in household contact with children

≤ 5 years and individuals 6-17 years were more frequently colonized than adults in household contact with just young children. Children ≤ 5 years who lived with 6-17 year-olds and adults carried more PCV serotypes than children living with just adults.
Conclusions: Age was the most important predictor of pneumococcal colonization at the individual level, and the presence of different age groups within households was the central predictor of household-level colonization. The serotype distribution of colonizing pneumococci will differ by age group, and the presence of children, adolescents, and adults in households. Further understanding about age-related immunity to colonization will be central to anticipating changes in the pneumococcal population due to introduction of PCVs in young children.

Introduction

Streptococcus pneumoniae is the leading cause of bacterial infections worldwide and has the potential to cause both mucosal and invasive infections. Invasive pneumococcal disease (i.e., pneumonia, meningitis, and sepsis) is a major cause of hospitalizations and mortality in children and the elderly globally (2). *S. pneumoniae* is part of the normal human nasopharyngeal (NP) flora, and hosts usually remain asymptomatic despite colonization. Disease occurs when the bacteria breach host mucosal barriers and invade sites, such as the middle ear, lungs, blood, or cerebrospinal fluid. Understanding the factors that lead to acquisition and carriage of pneumococci is crucial because colonization is a precondition for pneumococcal disease and asymptomatic colonization is the mechanism by which the majority of person-to-person transmission occurs (3, 24).

Previous studies have identified individual-level risk factors for pneumococcal carriage among children; these factors include young age, attendance at daycare centers, number of siblings, and recent respiratory tract illness. However, these individual-level risk factors are not able to fully account for the marked differences in NP carriage seen across communities (8, 10, 11). A small number of studies have illustrated that pneumococci are easily transmitted within families and between households within communities, highlighting the potential central role of community- and household-level characteristics in pneumococcal transmission (10, 12, 21). Owing to the contagiousness of infectious diseases, one would anticipate pneumococcal transmission to be associated with risk factors related to both an individual and the individual's close contacts (10, 16, 17, 20).

The introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in young children has renewed interest in pneumococcal transmission dynamics and understanding the colonization state, since the PCV7 reduces acquisition and density of colonization with vaccine-targeted serotypes (22). By reducing transmission of vaccine-targeted serotypes, the use of PCV7 in young children also lead to a herd affect, reducing colonization and disease due to vaccine serotypes among non-vaccinated members of the population. The population-wide benefits gained by PCV7's effect on colonization may be at least partially offset by the increases in non-vaccine-targeted serotypes in carriage and disease that have been documented subsequent to vaccine introduction (9, 18, 19, 22, 28).

The use of PCV7 has drawn attention to the central role of colonization in understanding pneumococcal epidemiology, examining population-level factors

associated with colonization, and anticipating how altering the colonization state with a conjugate vaccine may alter pneumococcal disease patterns. In the current study, we investigated both individual and group-level factors associated with pneumococcal colonization among healthy individuals of all ages residing in households in one neighborhood in Vietnam prior to vaccine introduction. We show that age was the most important predictor of pneumococcal colonization at the individual level, and the presence of different age groups within households was the central predictor of household-level colonization. We discuss these results in terms of the current understanding of the immune response to pneumococcal colonization and postulate how age-related immune mechanisms to colonization may shape pre- and post-vaccine serotype distributions among all individuals.

Methods

Study design and survey

We conducted a cross-sectional study of nasopharyngeal (NP) carriage in Phu Vinh hamlet of Vinh Thanh commune, Nha Trang district, Vietnam, in October, 2006. We randomly selected households in Phu Vinh with at least one child ≤ 5 years and, separately, households with only adults (≥ 18 years) from a detailed census list that was compiled by the Khanh Hoa Provincial Health Service prior to this carriage study. Trained interviewers recruited households until 75 households with children and 40 households with adults were enrolled. All currently residing household members were invited to participate in a one-time NP swab procedure and survey interview. A total of 146 households were approached, and 115 households participated (79%). Ninety-six

households with children were approached, and 75 households agreed to participate (78%). Fifty households containing only adults were approached, and forty agreed to participate (80%). The most common reason for refusal was that all household members were not available to participate, because they were employed far from home and had to travel extensively during the day.

All NP swab procedures and face-to-face interviews took place at the Vinh Thanh Commune Health Center. Trained interviewers asked adult participants about health behaviors (tobacco or dieu cay smoking, hand washing before eating and after bathroom use, use of any cleansing product when washing hands), health history (number of chronic health conditions, recent surgeries, respiratory symptoms or illness in the preceding week), recent antibiotic usage for respiratory and non-respiratory illnesses, and demographics (age, employment, income) using standardized questionnaires. Adult caregivers were asked similar questions about their children, including the child's breastfeeding history (current breastfeeding status, composition of diet if currently breastfeeding, ever breastfed, duration of breastfeeding if ever breastfed), child or daycare attendance (for children 0-5 years, including the type of childcare service currently attended and the number of hours spent at daycare during an average week), and school attendance. At the time of the interviews and swab procedures, three households which previously contained only adults presented with a small child (i.e., a female household member had given birth in the time between recruitment and survey). These three households were considered to contain one child $\leq$ 5 years for purposes of analysis. Altogether, 78 households contained at least one child $\leq$ 5 years, and 37 households contained only adults ($\geq$ 18 years).

Trained physicians obtained a single NP swab specimen from all participants, in accordance with World Health Organization recommendations (23). NP samples were obtained with calcium alginate-tipped wire swabs (Calgiswab® Type 1, Puritan Medical Products Company LLC, Guilford, ME). Swabs were passed through the anterior nares to the back of the nasopharynx, left in place for approximately two seconds, and rotated 180 degrees before removal. After collection, all NP swab specimens were immediately inoculated into STGG transport media and held at 4°C for no more than four hours at the Commune Health Center. The NP swabs were then transported to the local hospital, where they were stored at -20°C for one week. NP swabs were shipped on dry ice to the University of Michigan and stored at -80°C for one month prior to analysis (23).

Informed consent was obtained from all adults (≥ 18 years) and parents of children prior to participating in the investigation. Oral assent was also obtained from participants 6-17 years old prior to the NP swab procedure. All study procedures were approved by the Health Sciences Institutional Review Board (IRB) at the University of Michigan (HUM00006257), the IRB of the National Institute of Hygiene and Epidemiology in Hanoi, Vietnam, and the IRB of the International Vaccine Institute in Seoul, Korea (2006-009).

Bacterial culture and isolation

Frozen vials of STGG medium containing NP swab specimens were warmed in a 37°C water bath for 20 minutes and then vortexed for 20 seconds. Thawed samples in media (50 µl) were inoculated on trypticase soy agar with 5% sheep blood (TSA II) containing gentamicin (2.5 mg/L) and separately on non-selective media (TSA II). All

specimens were incubated at 37°C in 5% CO_2 overnight (O/N). Presumptive identification of pneumococcal colonies was based on colony morphology and α-hemolysis on blood agar (23). Up to 10 pneumococcal colonies were sampled per individual. If more than one pneumococcal colony morphology was present, then up to 10 colonies per morphology type were isolated.

Confirmatory identification of pneumococci was based on optochin sensitivity (zone ≥14 mm with 6mm P discs after O/N incubation at 37°C in 5% CO_2), solubility in 10% deoxycholate using the plate method, and Gram stain (23).

Capsular typing

DNA was extracted from each pneumococcal isolate as previously described, and the Latin American formulation of a sequential multiplex PCR was used for serotyping (7, 25). Each PCR reaction included *Streptococcus pneumoniae* ATCC® 49619 (serotype 19F) as a positive control and a no-template (dH_2O) negative control. PCR products were electrophoresed on 2% NuSieve® GTG® agarose gels in TAE buffer at 125-128 V for 38 minutes. Gels were stained in ethidium bromide (0.5 μg/mL), and PCR product sizes were compared to a 50-bp molecular standard (Novagen, Inc). A 20% random selection of PCR-designated serotypes was confirmed with latex agglutination followed by the Quellung reaction (Statens Serum Institut, Denmark). The presence of newly-identified serotype 6C was investigated among serotype 6A isolates by use of a multibead assay based on monoclonal antibodies and multiplex PCR (Moon Nahm, MD, personal communication) (29).

Nontypeable pneumococci

Any isolate that was optochin sensitive and bile soluble but could not be typed with either the multiplex PCR or antisera was considered to be nontypeable (NT). The Gen-Probe® AccuProbe® pneumococcus culture identification test (Gen-Probe, Inc., San Diego, CA), a DNA probe hybridization test based on the rRNA gene sequence, was used to confirm the identity of all NT isolates (1, 4, 6).

Statistical analyses

All statistical analyses were performed with SAS v 9.1.3 (SAS Institute, Cary, NC). The Cochran-Armitage test for trend was used to check for a linear decrease in the frequency of pneumococcal colonization with increasing age. A Mantel-Haenszel chi-square test was used to compare the frequency of colonization across age groups in households with and without individuals 6-17 years. From cross tabulations, the frequency of colonization among different age groups in households with and without 6-17 year olds and the frequency of carrying PCV vs. non-PCV serotypes among children 0-2 and 3-5 years were assessed using chi-square statistics or Fisher's Exact Test when within cell sizes were small; odds ratios (ORs) and 95% confidence intervals (CIs) were also generate to asses the magnitude of the associations. Heterogeneity in the odds ratios comparing the frequency of carrying PCV vs. non-PCV serotypes in children 0-2 vs. children 3-5 years, stratified by daycare attendance, was assessed with the Breslow-Day chi-square test. Generalized estimating equations (GEEs) specifying an exchangeable correlation matrix were used to examine the statistical relationships between individual-level characteristics and the likelihood of pneumococcal colonization and, separately, the

relationships between household-level characteristics and the proportion of household members colonized. GEEs were also used to ORs and 95% CIs to assess the magnitude of the associations between individual-level characteristics and pneumococcal colonization. Any household-level characteristic that was statistically associated ($p<0.05$) with household-level pneumococcal colonization in the univariate analyses was entered into a multivariate GEE model to investigate the statistical relationship between household-level characteristics and household-level pneumococcal colonization, while controlling for other variables.

Results

Carriage and serotype distribution of *S. pneumoniae* by age

Eleven percent of 519 individuals (n=56) were colonized with *S. pneumoniae*. The frequency of pneumococcal carriage was greatest among young children and decreased with age ($p<0.0001$, Table 6.1).

The serotype distribution of colonizing pneumococci varied by age. Young children were more likely to carry serotypes targeted by one of the pneumococcal conjugate vaccines (PCVs), while older children and adults were more likely to carry non-PCV-targeted serotypes and nontypeable pneumococci (see Table 3.1 of thesis Chapter 3). Further examination of the serotypes carried by children 0-5 years suggested that children ≤ 2 years (n=18) may be more likely than children 3-5 years (n=13) to carry PCV-targeted serotypes, although this relationship was not statistically significant (OR: 8.00, 95% CI: 0.78, 82.46) (Figures 6.1-6.2). There was no difference in the frequency of carrying PCV-targeted pneumococci among children 0-2 and 3-5 years who attended

daycare (100% vs. 67%, respectively) compared to children 0-2 and 3-5 years who did not attend daycare (91% vs. 100%, respectively) (χ^2_{BD} p=0.20).

Carriage and serotype distribution of *S. pneumoniae* by household composition

The frequency of pneumococcal colonization varied by household composition. The frequency of pneumococcal colonization was the same for children 0-5 years in households containing individuals 6-17 years compared to households without older children and adolescents, but adults in household contact with children 0-5 years and 6-17 years were more likely to be colonized than adults in household contact with just children 0-5 years (p=0.06, based on Fisher's Exact Test) (Table 6.2). Fifteen percent of all individuals in households with older children and adolescents were colonized, while 11% of all individuals in households without individuals 6-17 years carried pneumococci (χ^2_{MH} p=0.09).

The distribution of serotypes carried by household members may differ by the presence of different age groups. Children 0-5 years who lived with children aged 6-17 years and adults (n=18) were more likely to carry PCV-targeted serotypes than children 0-5 years who lived with just adults (n=13), although this relationship was not statistically significant (OR: 2.18, 95% CI: 0.31, 15.29) (Figures 6.3-6.4). There was no statistically significant difference in the frequency of daycare attendance among children 0-5 years in households with 6-17 year-olds and adults compared to 0-5 year-olds in households with just adults (56% vs. 71%, respectively, p=0.36). There was also no statistically significant difference in the frequency of 0-2 year-olds in households with 6-17 year-olds and adults (n=18) compared to 0-2 year-olds in households with just adults

(n=13) (67% vs. 50%, respectively, p=0.34). Colonized individuals who were 6-17 years old (n=6 individuals, 6 strains) carried nontypeable pneumococci (50%), non-PCV7 serotypes (33%), and a single PCV serotype (17%). Adults in households with 6-17 year olds (n=7 individuals, 7 strains) had the following distribution: 23F (14%), 14 (29%), 15B/C (14%), NT (43%). Adults in households with just small children (n=1 individual, 1 strain) carried a serotype 34 pneumococcus.

Individual and household-level characteristics associated with colonization

Among adults, no health behavior, health history, or demographic variables were associated with pneumococcal colonization, most likely due to the low prevalence of colonization in this age group. Among all children and adolescents < 18 years, having one or more respiratory symptoms in the seven days prior to the survey was associated with pneumococcal colonization (OR: 2.45, 95% CI: 1.25, 4.83). No other health behavior, health history, or demographic variables were associated with colonization among older children and adolescents 6-17 years, again due to the low frequency of colonization in this age range. Among young children ≤ 5 years, only attending child care was associated with an increased likelihood of colonization (OR: 2.55, 95% CI: 1.15, 5.66).

Tables 6.3-6.4 show the association of household-level variables with household-level pneumococcal colonization (the proportion of household members colonized). In the single variable analyses, the number of children 0-5 years in households, the number of older children and adolescents 6-17 years in households, and the total number of individuals within households were independently associated with the proportion of

household members colonized (Table 6.3). The number of adults within households, the number of children 0-5 years attending daycare and residing in households, the number of smokers in households, and household crowding were not associated with household-level colonization. The number of children 0-5 years in households, the number of older children and adolescents 6-17 years, and household size remained significant predictors of household-level pneumococcal colonization in a multivariate statistical model (Table 6.4).

Discussion

We investigated individual and household-level characteristics associated with pneumococcal colonization among people of all ages in Nha Trang, Vietnam. At both the individual and household levels, age was the most consistent predictor of pneumococcal colonization. The frequency of individual-level colonization was high among children ≤ 5 years, was greatest in three year-olds (67%), and then declined to approximately 2% among adults.

Larger household size is usually considered to be associated with an increased frequency of pneumococcal colonization among household members; this thought is based on the assumption that larger household sizes are due to an increased number of young children in the household or due to an overall level of crowding (5). In the current study, the proportion of household members colonized was positively associated with the number of children 0-5 years in households, after controlling for the presence of individuals 6-17 years and overall household size. Similarly, the proportion of household members colonized was positively associated with the number of older children and

adolescents 6-17 years in households, even when the number of children 0-5 years in households and household size were held constant. After taking into account the number of children 0-5 years and 6-17 years in households, household size was associated with a decrease in the proportion of household members colonized. This inverse relationship between household size and the proportion of household members colonized is most likely due to the large number of adults in the households in Vietnam; that is, after controlling for the presence of children < 18 years in households, larger household size is due to an increased number of adults. Since adults are least likely to be colonized of all age groups, arguably due to some form of immunity to colonization, a greater number of adults within households results in a greater number of immune individuals to potentially block within household transmission of pneumococci. Thus, while larger household size is usually assumed to be associated with an increased risk of pneumococcal colonization, large household size will be associated with less pneumococcal transmission in contexts where increasing household size is due to a larger number of adults and not an increasing number of children.

The distribution of carried serotypes also appeared to change with age. Adults and 6-17 year-olds more frequently carried non-PCV-targeted serotypes compared to children 0-5 years. A closer examination of the serotypes carried by this youngest age group who has the greatest prevalence of colonization suggested that children 0-2 years might be more likely than children 3-5 years to carry PCV-targeted serotypes; this potential relationship was not impacted by the frequency of daycare attendance among these age groups. Focusing on the household level, the presence of different age groups also may have affected the prevalence of colonization and the serotype distribution

carried by the different age groups within households. We examined a subset of our households that contained individuals who were 0-5 years, 6-17 years, and adults and compared them to households that contained only children 0-5 years and adults. Adults in household contact with children 0-5 and 6-17 years were more frequently colonized than adults in household contact with just children 0-5 years. This difference in colonization prevalence was not attributable to an increased number of small children in households with 6-17 year-olds, as the average number of children ≤ 5 years and the average number of children ≤ 2 years was the same for both types of households. Additionally, the serotype distribution may be different among children 0-5 years in household contact with 6-17 year-olds and adults compared to young children in household contact with just adults. Children 0-5 years living in households with 6-17 year-olds and adults appeared more likely to carry PCV-targeted serotypes compared to children 0-5 years living in households with just adults, and this variation was not attributable to differences in daycare attendance or the number of children 0-2 years in the two different types of households. We were not able to quantitatively compare the serotype distributions carried by adults in households with children 0-5 years and 6-17 years to that carried by adults in households with just children 0-5 years, due to the low prevalence of colonization among this age group.

It is interesting to speculate on the potential roles of different age groups in the transmission of pneumococci that are suggested by our observations. While colonization among 6-17 year-olds was approximately 12%, perhaps members of this age group in households with young children act as a bridge between young children and older household residents, functioning to spread pneumococci throughout the household. Older

children and adolescents may also acquire pneumococci from social networks outside of the home and introduce them to young and older individuals in households. Having been exposed (and presumably developed at least some immunity) to serotypes commonly carried by young children, older children and adolescents may also be susceptible to colonization with other serotypes and propagate these pneumococci among susceptible family members. In the current study, individuals who were 6-17 years old most frequently carried nontypeable (NT) pneumococci, followed by non-PCV-targeted serotypes, and then a single PCV-targeted serotype. Adults in household contact with small children and older children and adolescents carried a mixture of PCV serotypes, non-PCV serotypes, and NTs. When looking at all individuals within households, those with 6-17 year-olds carried more PCV serotypes overall, and households without 6-17 year-olds carried more non-PCV serotypes overall. Perhaps adults in household contact with only small children are quickly exposed and become immune to serotypes carried by the young children and therefore are susceptible to colonization with non-PCV serotypes. If adults in household contact with only small children are more likely to be immune to PCV serotypes, they may serve as a source of non-PCV serotypes to be transmitted to small children. Older children and adolescents in households may prevent some of the infectious contacts between young children and adults, delaying the exposure of adults to serotypes carried by young children in the household. Taken together, the presence of older children and adolescents in households was potentially linked with a greater prevalence of pneumococcal colonization adult household members, and, while 6-17 year-olds and adults were more likely to carry non-PCV7 serotypes and NTs compared to young children, there may be an overall higher prevalence of PCV serotypes carried

among household members in contact with small children, older children, and adolescents. While we are unable to comment on the independent contribution of adolescents to population-wide colonization (because we did not sample households with just adolescents and adults), adolescents in household contact with children and adults likely alter within household transmission of pneumococci and may be a source of outside introduction of pneumococci.

The decline in colonization with age and the potential contributions of different age groups to household-level pneumococcal colonization point toward the fundamental importance of understanding age-related immunity to colonization and the role of different age groups in pneumococcal epidemiology. It has been thought that the immune response to pneumococci in naturally exposed, unvaccinated children depends primarily on antibody directed against the polysaccharide capsule. Despite the suggestion that anticapsular antibodies play a role in the development of immunity to pneumococci, there is little evidence showing that antibodies are the primary mechanism of naturally acquired immunity, and several studies have suggested otherwise (27). Furthermore, the introduction of PCVs in young children are shifting the dynamics of the pneumococcal population, and attempts are being made to anticipate the post-PCV structure of the pathogen population, which serotypes may dominate following introduction of a PCV in young children, and the source of replacement strains in a vaccinated population. Here, it becomes apparent that a deeper understanding of the age-related immune response to colonizing pneumococci, of which there are over 90 known capsular types and nonencapsulated variants, is vital to these predictions. It has been shown that the frequency of pneumococcal colonization decreases with age and older children and adults

may carry a different distribution of pneumococcal serotypes compared to children 0-2 years, potentially serving as a source of replacement serotypes (see Thesis Chapter 3). However, it is also possible that the density of colonizing organisms and/or duration of colonization is reduced in adolescents and adults due to immunity, resulting in the appearance of a decreased frequency of colonization among older age groups, due to a failure of sampling techniques. Perhaps the "turn over" rate of colonizing pneumococci is faster among older children and adults with some immunity to certain serotypes due to previous colonization events. Adolescents and adults may briefly carry serotypes usually found among young children, but carry non-pediatric serotypes (to which they have not been previously exposed) for a longer duration. These postulations emphasize that predicting post-PCV pneumococcal antigen structure will require a greater understanding of the actual basis of the immune mechanisms that drive carriage, the duration of immunity to colonization, and how the density and duration of colonization may change with age. It will also be vital to elucidate how cross-immunity between strains will affect serotype distribution in all age groups. Partial cross-immunity has been demonstrated among some pneumococcal serogroups (e.g., 6, 23), but not others (e.g., 19). The pattern of dominating serotypes following PCV introduction may be greatly influenced by the strength of cross-immunity between serotypes and how the basic reproductive number (R_0) of one serotype may affect other serotypes.

After an examination of individual and household-level factors that affect pneumococcal colonization, we have shown that age is the strongest predictor of colonization at both the individual and contextual level and that the serotype distribution of carried pneumococci may vary by age group and network of contacts. These

observations motivate numerous fundamental questions about the age-related immune response to colonizing pneumococci which are yet to be answered. Understanding the exact mechanisms that drive immunity to carriage, the duration of colonization and the duration of immunity to colonization, how the density of colonizing organisms may be affected with immunity, and the degree of cross-immunity between strains will all determine the pneumococcal population structure prior to and following introduction of a PCV. It is important to note that the current study employed statistical procedures with underlying assumptions about the independence of infection outcome in individuals and a static correlation between individuals within households, both of which are known to be inappropriate for capturing the dependencies that occur with infection transmission. Mathematical models are being used increasingly in epidemiology, due to the recognition of the central importance of identifying and estimating the parameters that underlie the infection transmission process and the acknowledgment that the assumptions underlying standard epidemiologic data analysis techniques make them inappropriate for analyzing population systems that are characterized by non-linear feedbacks and dependency among outcomes in individuals (13-15, 26). Recognizing that solutions to the above-proposed questions about immunity will require years of epidemiologic, microbiologic, and immunologic research, future studies may benefit from the use of mathematical models to focus and test hypotheses and identify specific data to acquire. Thus, addressing these fundamental questions about the role of different age groups will require a focus on the serotypes carried by all age groups, the degree of contact between age groups, the age-related immune response to carriage, and use of appropriate survey and

microbiologic sampling and mathematical techniques to fully address how changing the pneumococcal population with a PCV will affect pneumococcal epidemiology.

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Figure 6.1: Percent serotype distribution among colonized children 0-2 years (n=19 encapsulated pneumococci isolated from 18 children) from household survey of pneumococcal colonization in Nha Trang, Vietnam, October, 2006

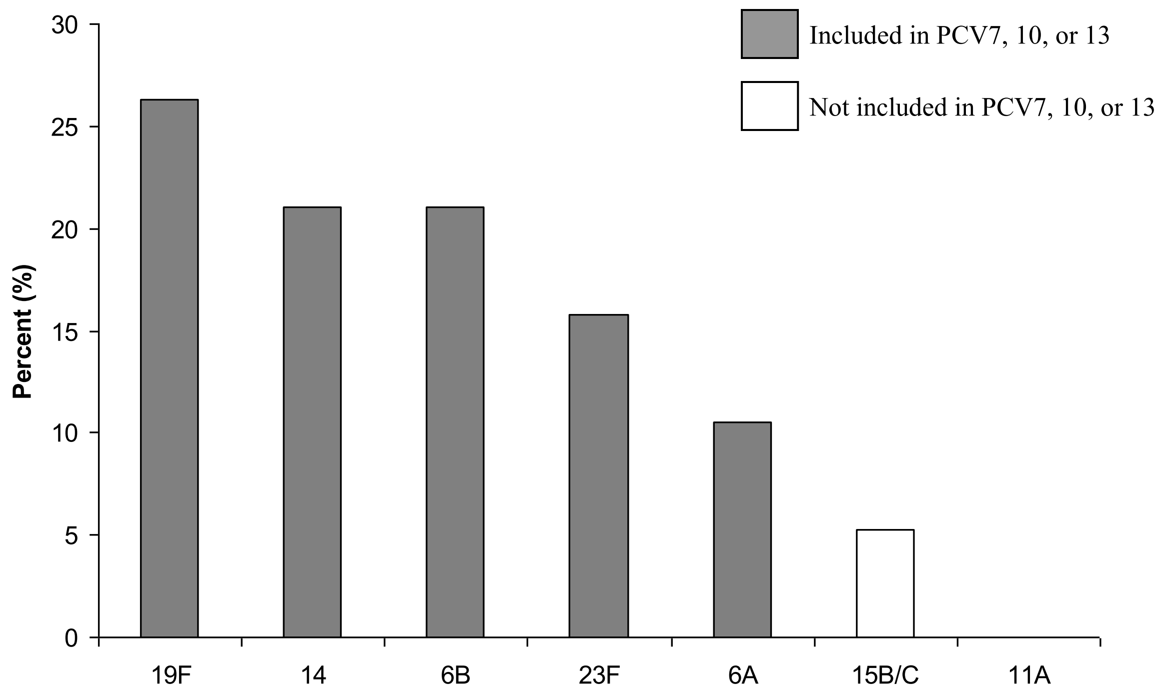

Figure 6.2: Percent serotype distribution among colonized children 3-5 years (n=13 encapsulated pneumococci isolated from 13 children) from household survey of pneumococcal colonization in Nha Trang, Vietnam, October, 2006

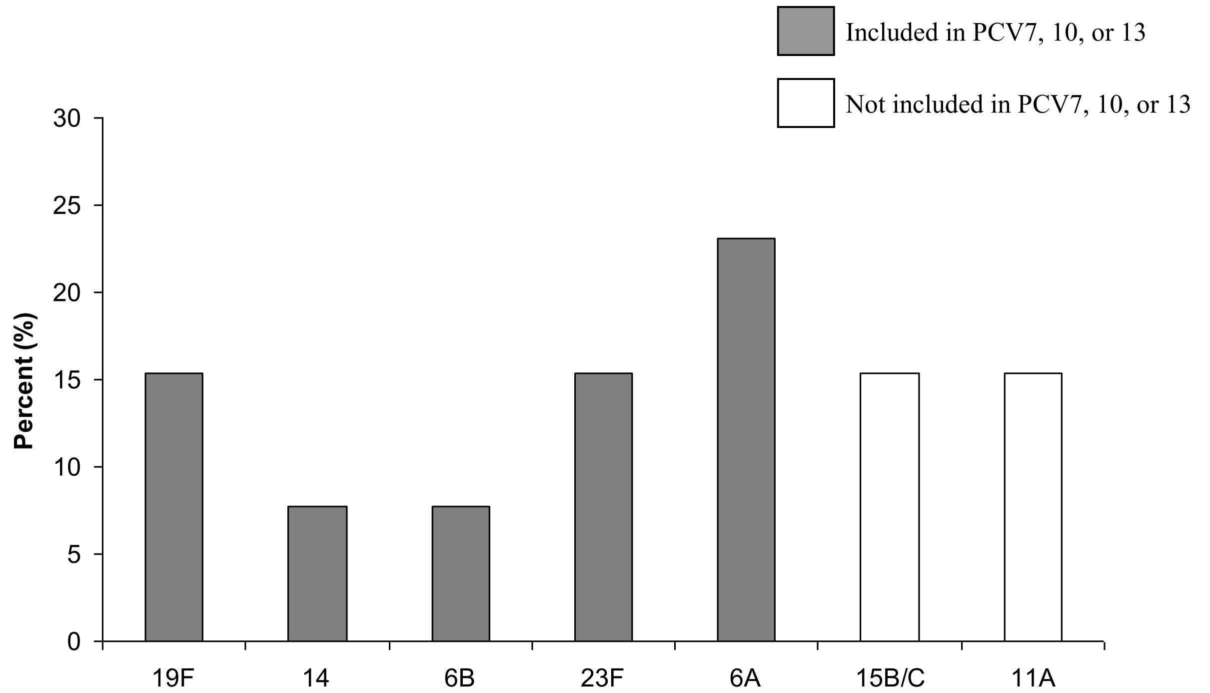

Figure 6.3: Percent serotype distribution among colonized children ≤ 5 years living in households with individuals 6-17 years and adults (n=18 encapsulated pneumococci isolated from 18 children) from household survey of pneumococcal colonization in Nha Trang, Vietnam, October, 2006

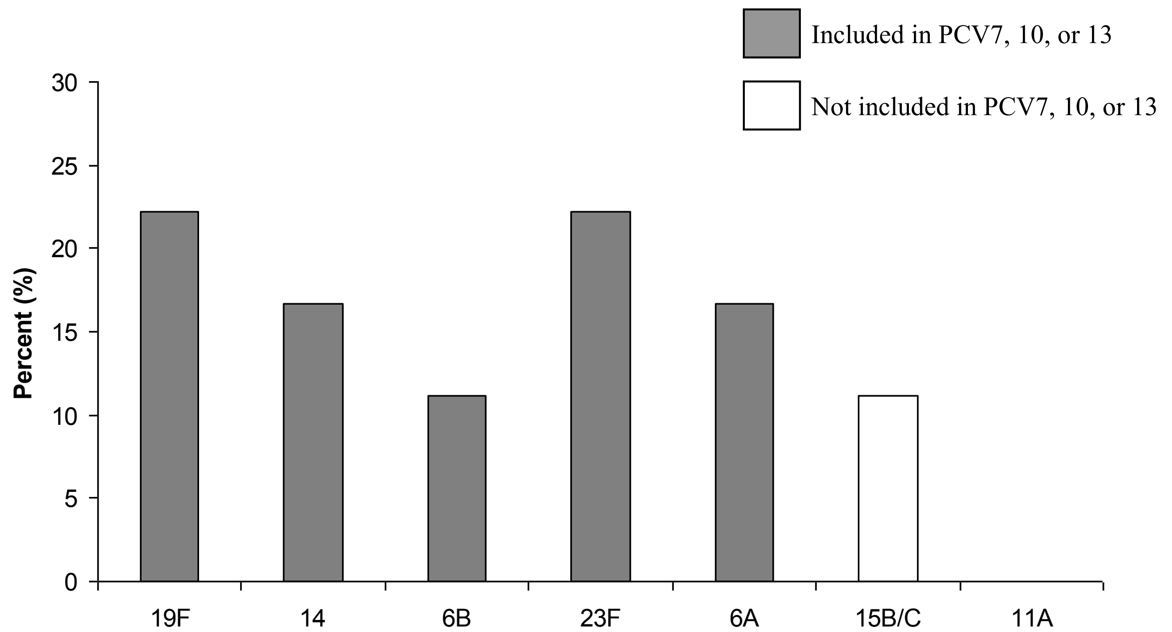

Figure 6.4: Percent serotype distribution among colonized children ≤ 5 years living in households with just adults (n=14 encapsulated pneumococci isolated from 13 children) from household survey of pneumococcal colonization in Nha Trang, Vietnam, October, 2006

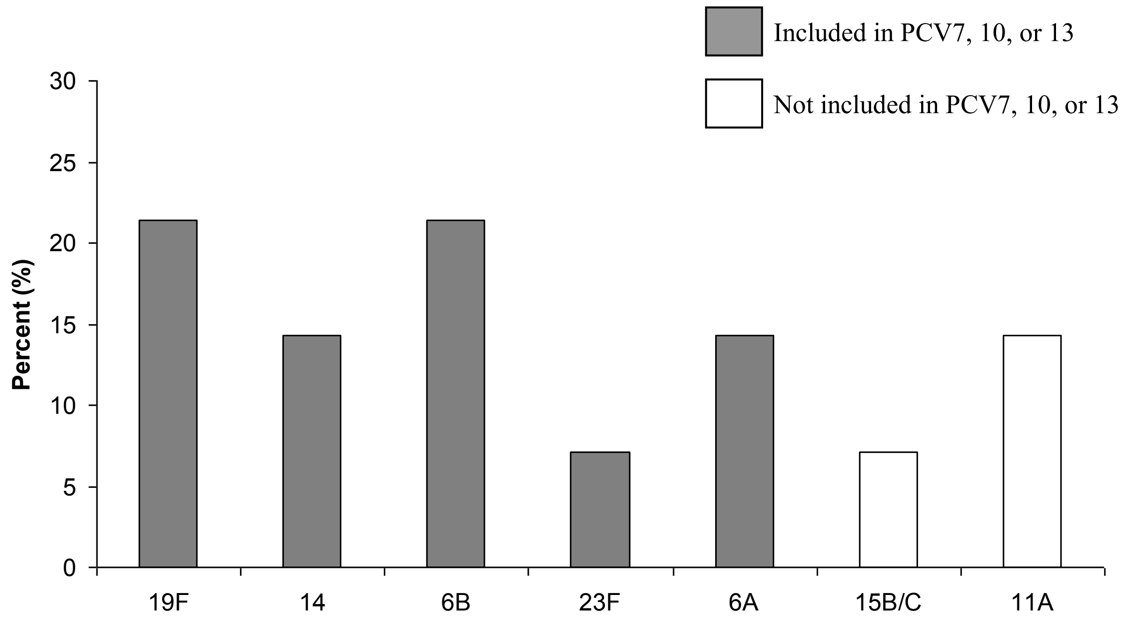

Table 6.1: Percent of each age group that was colonized with *S. pneumoniae* among 519 individuals participating in household survey in Nha Trang, Vietnam, October, 2006.

Age (years)	N	No. (%) Colonized	p-value†
≤ 1	41	14 (34)	<0.0001
2	19	7 (37)	
3	18	12 (67)	
4	15	7 (47)	
5	11	2 (18)	
6-10	22	3 (14)	
11-17	33	3 (9)	
18-30	142	2 (1)	
31-49	120	4 (3)	
≥ 50	98	2 (2)	
Total:	519	56 (11)	

†Cochran-Armitage Trend Test

Table 6.2: Frequency of pneumococcal colonization among age groups in households (HH) with vs. without older children 6-17 years

Age group (years)	HH with older children (N)	No. (%) colonized in HHs with older children	HH without older children (N)	No. (%) colonized in HHs without older children	OR (95% CI)	χ^2 p-value	χ^2_{MH} p-value
0-5	49	22 (45)	55	20 (36)	1.43 (0.65, 3.13)	0.38	0.09
6-17	55	6 (11)	0	–	–	–	
≥ 18	126	6 (5)	136	1 (0.7)	6.75 (0.80, 56.87)	0.06†	
Total	230	34 (15)	191	21 (11)			

†Fisher's Exact Test

Table 6.3: Univariate analyses predicting the proportion of household members colonized with *S. pneumoniae* (based on 519 individuals within 115 households)

Characteristic	β estimate	Standard error	p-value
No. of children 0-5 years in household	0.07	0.02	**<0.0001**
No. of children 6-17 years in household	0.04	0.02	**0.04**
No. of adults in household	-0.01	0.01	0.38
No. of children 0-5 attending daycare in household	0.06	0.03	0.07
No. of smokers in household	–0.01	0.01	0.58
Total no. of individuals in household (household size)	0.01	0.01	**0.04**
Household crowding (defined as the usual no. of residents in the household divided by the no. of rooms in the household)	0.03	0.02	0.17

Table 6.4: Multivariate analyses predicting the proportion of household members colonized with *S. pneumoniae* (based on 519 individuals within 115 households)

Characteristic	β estimate	Standard error	p-value
No. of children 0-5 years in household	0.11	0.03	**<0.0001**
No. of children 6-17 years in household	0.04	0.02	**0.03**
Total no. of individuals in household (household size)	-0.03	0.01	**0.01**

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Chapter 7

Conclusions and Future Directions

The overarching goal of this dissertation was to describe the epidemiology of colonizing *Streptococcus pneumoniae* among individuals in Vietnam, with the objective of providing the necessary data upon which health policymakers in this country could base a rational decision about an appropriate pneumococcal conjugate vaccine (PCV) for infants and children. By including individuals of all ages in our studies, we also more broadly aimed to shift the focus of infectious disease epidemiology from individual-level risk assessment to a systems approach to epidemiology that centers on the transmission of infectious agents through a population and how the role of different age groups and contact networks will shape disease patterns.

Chapter 3 of this dissertation showed that pneumococcal colonization is common among young, healthy children in Vietnam and that the frequency of colonization decreased with age. Children ≤ 2 years were more likely to carry PCV-targeted serotypes, while older children and adults more commonly carried non-PCV-targeted serotypes and nontypeable (NT) pneumococci. We found that antibiotic resistance was high among all colonizing pneumococci carried by all age groups, especially for penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole, and 66% of pneumococci were multidrug resistant. Based on the serotype distributions of colonizing pneumococci, we recommended use of PCV10 or PCV13, which would target 79% of

encapsulated pneumococci carried by young children in Vietnam. However, older children and adults may serve as a source of replacement serotypes following PCV introduction. Furthermore, non-PCV serotypes and NT pneumococci were also found to be highly resistant to various antibiotics and can serve as a reservoir of resistance determinants in pneumococci.

This study is one of a small number of carriage studies to include adolescents and adults and is the only study of colonization to include these age groups from an Asian country. As we have demonstrated the important role of these age groups in pneumococcal epidemiology, individuals of all ages should be included in future colonization studies, especially studies that aim to predict changes in pneumococcal serotype distribution following the introduction of a PCV in young children. A larger number of adolescents and adults than recruited in the current study will need to be included in the future, due to the lower prevalence of colonization among these age groups. Pre- and post-PCV longitudinal colonization studies of people of all ages could be most informative in terms of addressing the source of replacement serotypes in a population and how antibiotic resistance will evolve among pneumococci. Future household studies of colonization among people of all ages and their outside contacts (e.g., among all daycare attendees in the same facilities as the index children, coworkers of adults, etc) may be the most interesting. Future molecular work on isolated pneumococci would include multilocus sequence typing (MLST) to identify major clones circulating in Vietnam and to provide a better concept of the uniqueness of the isolates than just capsular type and antibiogram. Predicting the impact of a PCV on the evolution of antibiotic resistance also requires knowledge about the following bacterial-level

factors: 1) the mechanisms of antibiotic resistance; 2) the frequency of horizontal transfer of resistance determinants that occurs among populations of pneumococci, when under selective pressure from antibiotics and when antibiotics are absent; and 3) the frequency of capsular switching that happens among pneumococci, and whether the occurrence of capsular switching increases with PCV vaccination, given the demonstrated associations between capsular types and resistance profile. The degree to which antibiotic resistance evolves will also depend on antibiotic usage practices for respiratory and non-respiratory illnesses and the pharmacokinetics and pharmacodynamics of each antibiotic (and various other host and societal factors). Finally, the pre-vaccine prevalence of non-PCV serotypes presented in this chapter suggests the potential for replacement pneumococcal disease in Vietnam following PCV introduction, as has been observed in various other contexts. Introduction of PCVs in young children have also been associated with increases in otitis media caused by *Haemophilus influenzae* and *Moraxella catarrhalis* (1, 2). The limited valence of conjugate vaccines, their high cost, and concerns over replacement pneumococcal disease and disease caused by overgrowth of other microbiologic competitors of pneumococci in the human nasal cavity and nasopharynx suggest that conjugate vaccines that impact the colonization state may not be an ideal preventative strategy for pneumococcal disease. A renewed focus on pneumococcal vaccines that prevent disease but do not affect colonization should be considered.

The work presented in Chapter 4 of this thesis showed that NT pneumococci most likely constitute a larger proportion of the pneumococcal population than previously recognized and that NTs may be a considerable source of resistance determinants to the recombining pneumococcal population. Furthermore, older children and adults may be

more likely to carry NTs than encapsulated pneumococci, an observation which has implications for understanding the age-related immune response to pneumococci. All tested NT pneumococci appeared to lack the *cps* locus and were positive for *psaA*, while only two isolates were positive for *lytA*. Future work to confirm the identity and pathogenic potential of these isolates could include 16S rRNA gene sequencing, DNA-DNA reassociation experiments, MLST, further examination of the operon for capsule polysaccharide synthesis and investigation for the presence of the *aliB* ORF in place of the genes for capsule synthesis, and identification of the genes encoding IgA1 protease, pneumolysin, and complete sequencing of the *lytA* genes that can be detected. NTs may exhibit a greater variation in their *lytA* genes that have lead to LytA-like amidases that are less pathogenic, and further investigation into the allelic diversity of *lytA* genes from NT pneumococci is warranted to substantiate this theory. The existence of this segment of the pneumococcal population should be considered in microbiologic and human sampling strategies in future studies and vaccine design approaches.

Thesis Chapter 5 demonstrated that a recently developed multiplex PCR assay for capsular typing of pneumococci works for carriage isolates, in addition to its previously-established effectiveness with clinical isolates. The Latin American formulation of the multiplex PCR efficiently identified the serotypes found in Vietnam and should be used in other Asian countries with similar serotype distributions. This multiplex PCR assay for capsular genotyping is inexpensive and less subjective compared to conventional serotyping, and will be a valuable tool in pre- and post-PCV surveillance activities that focus on colonization. The next logical step for the multiplex PCR protocol is to expand the number of serotypes covered by the assay. A broader goal is to create a PCR-based

method of serotyping directly from transport media, i.e., without a culture step which is known to alter the relative prevalence of pneumococci and is most likely inefficient for quantifying multiserotype carriage.

In Chapter 6, we identified age as the most important predictor of pneumococcal colonization at the individual level, and the presence of different age groups within households was the central predictor of household-level colonization. The serotype distribution of colonizing pneumococci appeared to differ by age group and the presence of children, adolescents, and adults in households. These observations emphasize that further understanding about age-related immunity to colonization will be central to anticipating changes in the pneumococcal population due to introduction of PCVs in young children. Understanding the exact mechanisms that drive immunity to carriage, the duration of colonization and the duration of immunity to colonization, how the density of colonizing organisms may be affected with immunity, and the degree of cross-immunity between strains will all determine the pneumococcal population structure prior to and following introduction of a PCV. Thus, addressing these fundamental (and currently unanswered) questions about the role of different age groups will require a future focus on the serotypes carried by all age groups, the degree of contact between age groups, the age-related immune response to carriage, and use of appropriate survey and microbiologic sampling and mathematical modeling techniques to fully address how changing the pneumococcal population with a PCV will affect pneumococcal epidemiology.

In conclusion, we have extended the current research on pneumococcal epidemiology by focusing on all age groups, suggesting that NT pneumococci may

comprise a larger portion of the pneumococcal population than previously recognized and may increase in prevalence following introduction of PCVs, showing that a new multiplex PCR assay works for capsular typing of carriage isolates, and highlighting the need for further research particularly in the area of the age-related immune response to colonizing pneumococci. Specifically for Vietnam, we have shown that introduction of PCV10 or PCV13 has the potential to greatly benefit young children and may temporarily decrease the prevalence of antibiotic resistance, although replacement with non-PCV serotypes (especially serotypes 15B/C and 11A) that are also resistant will undoubtedly occur. Use of antibiotics and the introduction of the PCVs have changed the ecology of *S. pneumoniae*. While these public health interventions have undoubtedly saved thousands of lives, unintended consequences (such as replacement disease and the development of resistance) have occurred. Public health campaigns emphasizing judicious use of antibiotics are desperately needed, especially in Vietnam and other Asian countries. Ideal future pneumococcal vaccines should be multiserotype vaccines that prevent disease but not colonization, while still generating an immunologic response in children and infants 0-2 years. This approach will not generate a herd effect, but a vaccine that prevents serious pneumococcal disease while not eliminating pneumococci from the nasopharynx (as with protein vaccines that target all pneumococci and allowing for the potential growth of other pathogens, such as *Staphylococcus aureus*) should be considered in future research endeavors.

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