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The interaction between Streptococcus pneumoniae and other respiratory pathogens, including viruses and bacteria commonly colonizing the nasopharynx

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Abstract

The interaction between *Streptococcus pneumoniae* and other respiratory pathogens, including viruses and bacteria commonly colonizing the nasopharynx

By
Yu-Wen Chien

This dissertation includes three studies, each assessing the interaction of *Streptococcus pneumoniae* and other respiratory pathogens using different approaches. The first study assessed the importance of secondary bacterial infections, especially pneumococcal infections, in the 1918 influenza pandemic. A systematic review of antemortem cultures from normally sterile sites during the 1918 influenza pandemic was performed, showing that the majority of pneumonias and deaths were caused by secondary pneumococcal pneumonia. A meta-analysis of bacterial vaccine studies during the 1918 pandemic was also performed, suggesting that the efficacy of whole-cell killed pneumococcal vaccine was 59% (95% CI 43–70%) for prevention of pneumonia and 70% (95% CI 50% – 82%) for prevention of death. In the second study, a deterministic compartment model was developed to investigate the interaction of *S. pneumoniae* and influenza virus and the usefulness of antibacterial interventions in a future "1918-like" influenza pandemic. The model predicts that such a pandemic will result in many fewer deaths in current developed countries than in 1918 simply due to the decline in pneumococcal carriage and the herd immunity provided by the widespread use of pneumococcal conjugate vaccines. Antibiotic treatment of patients with secondary pneumonia can greatly reduce mortality; antibiotic prophylaxis will be less useful because the number needed to treat is too high. The findings of these two studies will help set up a more conservative upper bound on the disease burden of a 1918 – like influenza pandemic and have the potential to lead to substantial changes in pandemic planning.

The third study investigated the interaction of *Streptococcus pneumoniae, Haemophilus influenzae,* and *Staphylococcus aureus* in the nasopharynx using data from a longitudinal study in Peru. A positive association between *S. pneumoniae* and *H. influenzae* and a negative association between *S. pneumoniae* and *S. aureus* were found, no matter whether culture or real-time quantitative polymerase chain reaction (qPCR) was used to determine the colonization status. The densities of *S. pneumoniae* and *H. influenzae* were also positively correlated. These findings suggest that bacterial interactions in the nasopharynx are complex and thus vaccines and antimicrobials which target specific bacteria and may unexpectedly influence the bacterial flora.

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Chapter 1 - Introduction

Several bacterial pathogens, such as *Streptococcus pneumoniae, Haemophilus influenzae,* and *Staphylococcus aureus*, reside in the human upper respiratory tracts and are the source of respiratory infectious diseases including acute otitis media, sinusitis, and pneumonia. Among them, *Streptococcus pneumoniae* is the most important, and is the leading cause of bacterial respiratory infections throughout the world (1).

Respiratory viral infections have been implicated in the pathogenesis of infections, colonization and transmission of respiratory bacteria. For example, pneumonias cause by *Streptococcus pneumoniae* and *Staphylococcus aureus* have been suggested to be associated with preceding influenza infections (2, 3, 4, 5, 6). Several ecological studies have found a temporal correlation between incidence of invasive pneumococcal disease and several respiratory viruses, including influenza virus, respiratory syncytial virus and human metapneumovirus (7, 8, 9, 10). In addition to the increased risk of secondary pneumonia and invasive diseases, acute respiratory infections have also been associated with increased pneumococcal colonization in the human upper respiratory tracts (11, 12, 13), which is consistent with laboratory studies showing that cells pre-infected with RSV, influenza virus or adenovirus have higher adherence with *S. aureus*, *S. pneumoniae* and group B *Streptococcus* (14, 15, 16). Moreover, respiratory infections with rhinovirus, adenovirus and echo virus have been demonstrated to facilitate the spread of *S. aureus* and *S. pneumoniae* in humans (17, 18, 19, 20).

The strongest impact of this viral-bacterial interaction can be demonstrated in influenza pandemics. The first part of my thesis focused on the interaction of influenza virus and common respiratory bacteria, particularly *S. pneumoniae*, in influenza pandemics. I first sought to evaluate the importance of bacterial co-infection in the 1918 pandemic, the most catastrophic infectious event in history, through a systematic review of antemortem cultures from normally sterile sites and a meta-analysis of bacterial vaccine studies in 1918. In my second project, I developed a mathematical model to investigate the viral-bacterial interaction and the effect of antibacterial intervention in a severe "1918-like" influenza pandemic.

Nasopharyngeal bacterial colonization is an important step in the pathogenesis of respiratory infections and is also a source of transmission in the community. Understanding factors that influence bacterial colonization is essential. Previous studies suggest that the presence of micro-organisms in the nasopharynx can affect the colonization of other pathogens (11, 21, 22, 23, 24, 25, 26). My third project aimed to investigate the interaction of three common respiratory bacteria, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus*, in the nasopharynx using data from a longitudinal study in Peru.

Chapter 2 - Background information and review of literature

Streptococcus pneumoniae, or the pneumococcus, was first isolated independently by Louis Pasteur and George Sternberg in 1881 (27). It is a Gram-positive, alpha-hemolytic bacterium belonging to the genus *Streptococcus*. Pneumococcal cells are lancet-shaped cocci usually arranged in pairs or short chains. Individual cells are 0.5 to 1.2 μm in diameter (27). It has a polysaccharide capsule which is an essential determinant of virulence related to its invasive potential. The capsular polysaccharides have been used for serologic classification, and currently 93 different serotypes have been identified (28).

2.1. Epidemiology of *S. pneumoniae*

2.1.1. Disease burden caused by *S. pneumoniae*

S. pneumoniae has been known as the leading cause of otitis media, community acquired pneumonia, bacteremia, and meningitis throughout the world (29). In the United States, it was estimated that pneumococci account for approximately 3000 cases of meningitis, 500,000 cases of pneumonia and 7,000,000 cases of otitis media each year (30). The World Health Organization (WHO) estimated in 2005 that 1.6 million people, including 0.7 – 1 million children aged less than 5 years, die of pneumococcal disease every year, and the disease burden is highest in developing countries (31).

Young children (aged <2 years), the elderly (aged >65), patients with primary or acquired immunedeficiencies (e.g. HIV infection, sickle cell disease, splenectomy or asplenia), and people with comorbidities (e.g. congestive heart failure, diabetes

mellitus, asthma, alcohol abuse) are more likely to develop invasive pneumococcal disease (IPD) defined as isolation of pneumococci from normally sterile sites (usually blood, pleural fluid, and cerebrospinal fluid) (29). Some ethnic groups, such as indigenous population of Alaska, African Americans, American Indians and Australian aborigines, have also a higher risk of IPD, which may be due to both genetic factors and socioeconomic factors (29, 32).

2.1.2. Colonization

S. pneumoniae is part of the normal microbial flora of nasopharynx and the colonization is usually asymptomatic and transient. The prevalence of pneumococcal colonization depends mainly on age. A cross-sectional study of more than 3000 healthy children aged 0 – 19 years in The Netherlands showed that the prevalence of pneumococcal colonization gradually increases and peaks around 55% at the age of 3 years; the prevalence then gradually declined until a stable prevalence of 8% after the age of 10 years (22). The reported prevalence of nasopharyngeal colonization in young children ranges from 20 – 86%, usually higher in developing countries (1, 33). The prevalence in adults is much lower, ranging from 4 – 12% in developed countries (29, 34, 35, 36, 37), though a prevalence of 40% has been reported in one developing country (38). Having young siblings, day care attendance, acute upper respiratory infections, crowding, exposure to cigarette and asthma have been associated with higher prevalence of pneumococcal colonization (33).

S. pneumoniae is easily transmitted from person-to-person by respiratory secretions of patients or healthy carriers, either through inhalation of bacteria or indirectly via contact with contaminated surfaces (1, 39). Pneumococcal diseases occur only after

nasopharyngeal colonization with a homologous strain (33, 40). Therefore, nasopharyngeal colonization by the pneumococcus, though usually asymptomatic, is the main source of transmission in the community and is also an important step in the pathogenesis of pneumococcal infections.

2.1.3. Serotype distribution among IPD

Although there are 93 different serotypes of *S. pneumoniae*, only some of them commonly cause diseases in humans (28). The serotype distribution varies with age, time and geographic regions. Globally, about 20 serotypes accounts for >80% of IPD in all age groups (31). Serotypes 14, 4, 1, 6A, 6B, 3, 8, 7F, 23F, 18C, 19F, and 9V are the most important serotypes (29). In children, the serotype distribution is more limited and the 13 most common serotypes account for at least 70 – 75% of IPD (31). The predominant serotypes in children include serotype 6, 14, 18, 19 and 23F (29). Geographic difference in serotype distribution has been noted; for example, serotypes 1 and 5 are commonly isolated in developing countries but are uncommon in developed countries (29).

2.1.4. Pneumococcal vaccines

Vaccines are important measures to combat pneumococcal diseases, especially in this era of increasing antimicrobial resistance of pneumococci. Currently, there are two types of pneumococcal vaccines: pneumococcal polysaccharide vaccines (PPV) and pneumococcal conjugate vaccines (PCV).

2.1.4.1. Pneumococcal polysaccharide vaccines (PPV)

The 23-valent PPV is a capsular polysaccharide vaccine which contains 23 of the

most common serotypes, covering ~ 90% of IPD (41). It has been available for more than 20 years. Earlier studies showed the vaccine efficacy of PPV against IPD in healthy young adults is 70 – 80% (30). The most recent Cochrane Review concludes that the vaccine is 74% effective in preventing IPD in the elderly, but the vaccine efficacy in adults with chronic illness is still controversial. The evidence of its efficacy against all-cause pneumonia is also inconclusive (42). In the United States, the PPV is recommended for use in adults 65 years of age or older, people older than two years of age with high risk of disease (e.g. functional or anatomical asplenia or HIV infections or other immunocompromising conditions) and adults who smoke cigarettes or have asthma (43). The PPV has no effect on pneumococcal colonization in the elderly (29). Another limitation is that children aged < 2 years cannot mount a good immune response to polysaccharide vaccines (29).

2.1.4.2. Pneumococcal conjugate vaccines

Pneumococcal conjugate vaccines (PCV) in which capsular polysaccharides are conjugated to a protein carrier are more immunogenic in young children (29). A recent meta-analysis of randomized controlled trials concludes that the vaccine efficacy is 80% in preventing vaccine-type IPD in children less than 2 years of age (44). The vaccine protection effect lasts for at least 2 to 3 years and probably longer (31). The heptavalent PCV (PCV7) was introduced in the United States in February 2000. It was originally recommended for children aged 2–23 months and for children aged 24–59 months who are at increased risk for pneumococcal disease (e.g., sickle cell disease (SCD), HIV infection, and anatomic or functional asplenia) but in 2007 the recommendation was expanded to include all children aged 2- 59 months (45).

The PCV7 includes serotype 4, 6B, 9V, 14, 18C, 19F, and 23F which in total accounted for >80% of IPD in children in the United States before the introduction of PCV in 2000 (46). The routine use of PCV7 for childhood immunization has reduced the incidence of vaccine-type IPD among children <5 years old from 81.9 cases per 100,000 population in 1998-1999 to 0.4 per 100,000 in 2007. The incidence of overall IPD among children < 5 years old declined from 98.7 per 100,000 in 1998-1999 to 23.6 per 100,000 in 2007, a reduction of 76% (47). The IPD incidence decreased not only among children targeted for vaccination but also among unvaccinated adults and children, especially those aged ≥ 65 years among whom the overall IPD incidence decreased from 60.1 per 100,000 in baseline to 37.9 per 100,000 in 2007, a reduction of 37%. The overall IPD incidence in all age groups declined by 45% from 24.4 to 13.5 per 100,000 (47). This is because the PCV prevents not only pneumococcal disease but also asymptomatic colonization of the serotypes in the vaccines among young children who are the main reservoir of pneumococcal transmission, resulting in great herd immunity effect that protects unvaccinated population (48). Although the overall IPD incidence has greatly declined after the introduction of PCV7, the prevalence of pneumococcal colonization does not change because of serotype replacement with non-vaccine serotypes which in general are less virulent (48, 49). However, virulent serotypes not included in PCV7, such as serotype 19A, have emerged after the widespread use of PCV7 and partially offset the vaccine effects (47, 48). Therefore, a 13-valent PCV (PCV13) which includes 6 additional serotypes (serotypes 1, 3, 5, 6A, 7F, and 19A) was introduced in the United States in 2010 (50).

2.2. The interaction between influenza virus and respiratory bacteria, particularly the pneumococcus

Respiratory viral infections have been suggested to influence the transmission and pathogenesis of bacterial pneumonia in three ways: 1) increase bacterial acquisition and colonization in the upper respiratory tract; 2) increase transmission of colonized bacteria to other people; 3) increase the susceptibility of hosts to secondary pneumonia caused by colonizing bacteria.

2.2.1. Viral infections increasing bacterial acquisition

Several epidemiologic studies have shown that people with respiratory viral infections are more likely to be colonized with *S. pneumoniae* (11, 12, 13). A recent animal study shows that influenza – infected ferrets are more likely to acquire pneumococcal acquisition than influenza – free ferrets (51). These observations are consistent with *in vitro* studies showing cells pre-infected with RSV, influenza virus or adenovirus have higher adherence with *S. pneumoniae* and *S. aureus* (14, 15, 16).

2.2.2. Viral infections increasing bacterial transmission

It has been shown that some infants and adults who are colonized with *S. aureus* and simultaneously have respiratory viral infections become “cloud persons” who can disperse a large number of bacteria and are highly contagious (17, 20). A study in college students also shows that the dispersal of colonized *S. aureus* increased after experimentally infected with a rhinovirus (19). Transmission of the pneumococcus has been shown to be associated with respiratory viral infections (52). Influenza – infected ferrets are shown to transmit pneumococci more efficiently than influenza – free ferrets (51). Brundage hypothesized in 2006 that influenza infection increases the aerosolization of bacteria that colonize the respiratory tract, enhancing transmission to other people (53).

2.2.3. Viral infections increasing the susceptibility to bacterial pneumonia

Several studies suggest that pneumonias cause by *Streptococcus pneumoniae* and *Staphylococcus aureus* are associated with preceding influenza infections (2, 3, 4, 5, 6). Synergistic pathogenicity of influenza virus and bacteria, particularly hemolytic streptococci and pneumococci, has been demonstrated in animals. In 1935, Brightman used a ferret intranasal inoculation model to demonstrate that combined influenza and streptococcal infection were highly fatal, even though neither agent was pathogenic when administered alone (54). Recent studies in mice have shown a lethal synergism between the influenza virus and the pneumococcus: a non-lethal pneumococcal challenge 7 days after influenza infection leads to the rapid and dose-related death in mice (55). If the pneumococcal exposure is followed by influenza, there is no synergistic lethality (55). The increased susceptibility to pneumococci seems to be unrelated to the depletion of lymphocytes or polymorphonuclear neutrophil granulocytes (PMN) in mice (56). Madhi ***et al.*** recently summarized possible mechanisms for this synergistic effect of influenza virus and bacteria in 2008 as follows (15):

a. Influenza virus induces the destruction of respiratory epithelium, enhancing bacterial adherence by exposing basement membrane elements (fibrinogen).
b. Cytokines induced by influenza virus infection (IL-1 and TNF) up-regulate cell surface receptors (platelet-activating factor receptor), which interact with pneumococcal ligands.
c. Viral neuraminidase cleaves sialic acid from respiratory epithelium, exposing cell receptors for pneumococcal adherence.
d. Some strains of influenza virus expressing PB1-F2 protein can cause more severe

immunopathology of secondary bacterial pneumonia.

e. Influenza virus depresses chemotaxis and suppresses phagocytosis.

f. Influenza virus increases IL-10 and enhances susceptibility to pneumococcal pneumonia.

g. The increase of interferon γ caused by adaptive immune response to influenza virus infection down-regulates the macrophage receptors with collagenous structure (MARCO) on alveolar macrophages that are involved in phagocytosis of unopsonized pneumococci (57).

2.3. Bacterial co-infections in pandemic influenza

2.3.1. The importance of bacterial co-infection during the 1918 influenza pandemic

The 1918 influenza pandemic caused an estimated 50 – 100 million deaths worldwide (58) and is considered as the worse-case scenario for pandemic preparedness. In the US military camps, the influenza attack rate was reported to be 21%. Of these, 17% had pneumonia, with 34% mortality (59).

It is essential to understand the underlying factors that led to such an enormous death toll in order to reduce mortality from future flu pandemics. Current pandemic plans mainly focus on stockpiling of antiviral drugs and development of vaccines against novel strains of influenza virus based on the belief that a hypervirulent strain of influenza virus led directly to most of the deaths in the 1918 pandemic by causing a primary viral pneumonia and acute respiratory distress syndrome (ARDS), and that secondary bacterial pneumonias may not have had a significant role in mortality (53, 60, 61, 62, 63). The evidence to support this belief seems to come from anecdotal

stories of fulminant cases dying within hours after influenza onset (64) and the results of animal studies which show that the reconstructed 1918 influenza virus is highly pathogenic in mice and monkeys and can induce aberrant innate immune responses that lead to extensive damage in the lungs (65, 66, 67, 68).

However, pandemic plans exclusively focusing on influenza virus have several limitations. It takes at least 6 months to produce influenza vaccines after the isolation of a new virus (60). Antiviral resistance may develop after the mass use of antiviral drugs but the drug choice is limited. In addition, influenza vaccines and antivirals are not affordable to many developing countries. Therefore, preventive measures targeting on bacteria, such as bacterial vaccines and antibiotics, may be more cost-effective to combat future influenza pandemics if it can be proven that most deaths in the 1918 influenza pandemic are caused by secondary bacterial infections.

Although most of the contemporary reports emphasized the importance of secondary pneumonia as a major cause of death in the 1918 pandemic, these studies appear to have been long forgotten, perhaps because contemporary investigators were seeking a bacterial etiology for the epidemic and the influenza virus was only subsequently discovered in the 1933 (69). Thus the attention of researchers and policy makers for pandemic preparedness focuses disproportionally on the influenza virus alone. The contemporary reports and the role of secondary bacterial pneumonia in the 1918 pandemic have come to the attention of a few modern researchers through two different avenues of investigation.

In the first line of investigation, Brundage reviewed the original epidemiological data

from military sources (53) and more recently with Shanks (70) reviewed both military and civilian data from the 1918 pandemic. They summarized eight epidemiologic and clinical characteristics of the 1918 pandemic and suggested that it was the sequential infection of influenza and common colonizing respiratory bacteria that caused the highly fatal pneumonias in most victims (70).

Taking a postmortem histopathological approach, Morens *et al.* examined the recut lung tissue specimens from 58 influenza fatalities during the 1918 pandemic which showed convincing histological evidence of severe acute bacterial pneumonia in nearly all cases (71). They also extensively reviewed the pathologic records and postmortem bacteriologic data for 8398 fatal cases in the 1918 pandemic from 109 published autopsy series and found consistent evidence of secondary bacterial pneumonia in the great majority of fatal cases. Bacteria could be recovered from more than 90% of the lung cultures, roughly 80% of the pleural fluid samples, and approximately 80% of the blood specimens (mainly heart blood taken postmortem).

However, the epidemiologic and clinical evidences provided by Brundage *et al.* are only indirect evidence and cultures during autopsy may be subject to contamination or post-mortem over-growth. Therefore, these two studies could not provide a final conclusion on whether the majority of deaths in 1918 were caused by secondary bacterial pneumonia. Cultures from normally sterile sites from living patients are the "gold standard" to establish a bacterial etiology for pneumonia, with a stronger etiological significance than sputum cultures or cultures taken from tissues post-mortem. Therefore, a systematic review of antemortem cultures from normally sterile sites during the 1918 pandemic is essential to elucidate the major cause of

death in 1918.

2.3.2. Bacterial vaccines in the 1918 influenza pandemic

There are few modern studies that have evaluated the usefulness of bacterial vaccinations in influenza pandemics because influenza research has almost exclusively focused on influenza virus itself since the discovery of influenza virus in 1933. The etiology of influenza was unknown in 1918. Many contemporaneous investigators erroneously believed that bacteria, in particular *Bacillus influenzae* (Pfeiffer's bacillus, now known as *Haemophilus influenzae*) was the cause of influenza (72). Therefore, many bacterial vaccines were produced, tested, and administered during the 1918 pandemic in attempts to prevent influenza and to investigate the etiology of influenza, providing a background to evaluate the effect of bacterial vaccines in influenza pandemics. In addition, if bacterial vaccines are proved to be effective in reducing the risk of pneumonia or death, this will provide additional evidence to support the hypothesis that secondary bacterial pneumonia was important in 1918.

2.3.3. Modeling the interaction of influenza virus and bacteria and the effect of anti-bacterial interventions in influenza pandemics

There have been many studies evaluating the effect of different strategies to contain or mitigate severe influenza pandemics by modeling approaches (73, 74, 75, 76, 77, 78). To our knowledge, however, few of these models consider the synergistic effect of influenza virus and respiratory bacteria as well as the effect of antibiotic prophylaxis during influenza pandemics. My review of antemortem cultures from normally sterile sites in the 1918 pandemic showed that *Streptococcus pneumoniae* was the

predominant cause of influenza-associated pneumonia in the majority of the studies; in some geographic areas, it was the only pathogen isolated in antemortem cultures from sterile sites. The prevalence of pneumococcal colonization in adults was approximately 40% in 1910s (34, 35, 36), which was much higher than that in developed countries today, though a similar high prevalence may still be found in developing countries (38). Prevalence of pneumococcal colonization may influence the severity of an influenza pandemic by influencing the likelihood of secondary pneumonia. Given the current lower pneumococcal prevalence and widespread use of PCV, it is possible that even if a 1918-like influenza virus emerges, far fewer cases of pneumococcal pneumonia will occur in developed countries today than in 1918, even without antiviral drugs, influenza vaccines or other specific interventions.

Influenza vaccines and antivirals are not affordable to developing countries and antibiotics may be more cost-effective for pandemic preparedness. Antibiotic prophylaxis for influenza patients is generally not recommended in seasonal influenza because the risk of developing secondary pneumonia is low. However, in a 1918-like pandemic in which up to 17% of influenza patients may develop pneumonia as suggested by the military data (59), antibiotic prophylaxis for influenza patients may be justified to reduce the incidence and mortality of secondary pneumococcal pneumonia. Moreover, antibiotic prophylaxis may diminish the transmission of bacteria in the community and further reduce the incidence of secondary pneumonia. A mathematical model will help evaluate the effect of different pneumococcal prevalence and the effect of antibiotic prophylaxis on the severity of a future 1918-like influenza pandemic and provide new perspectives for pandemic preparedness.

2.4. Bacterial interactions in the nasopharynx

Nasopharyngeal colonization is an important step in the pathogenesis of respiratory bacterial infections, such as acute otitis media and pneumonia. The process of nasopharyngeal colonization is complex and not fully understood. Host factors, such as age, gender, immunity, and environmental exposure to tobacco smoke have been shown to be associated with bacterial colonization (79). Previous studies suggest that bacteria commonly colonized in the respiratory tract may interact with each other. For example, studies have shown a negative association between *Staphylococcus aureus* and *Streptococcus pneumoniae* (especially vaccine-type) (21, 22) and a positive association between *S. pneumoniae* and *Haemophilus influenzae* in the upper respiratory tract (11, 24). Understanding the how bacteria interact with each other in the nasopharynx, no matter whether it is synergistically or competitively, is essential for the design of preventive measures. This is especially true in this era of vaccines and antimicrobials which target specific bacteria and may unexpectedly increase the bacterial flora. For example, a negative association between colonization of *S. pneumoniae* and *S. aureus* has been suggested (21, 22). If this association is true and *S. pneumoniae* and *S. aureus* compete with each other in the nasopharynx, the immunization with pneumococcal conjugate vaccines (PCV) may result in higher prevalence of colonization of *S. aureus*. This is of great concern due to the increasing incidence of community-acquired methicillin-resistant *S. aureus* (MRSA) infections.

Previous studies examining the possible interaction of bacteria colonization are limited. In addition, previous studies only use traditional culture methods to determine the colonization status (presence/absence) of the bacteria. Real-time quantitative

polymerase chain reaction (qPCR) is a more sensitive tool to detect bacterial colonization and can quantify the density of bacteria in the nasopharynx. The density of bacteria colonization may influence the risk of infection and the probability of transmission to others. Therefore, using bacterial density instead of colonization status to assess bacterial interaction may provide new perspectives.

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Chapter 3 – Organization and objectives of the dissertation

3.1. Organization

This dissertation was divided into three separate studies, each assessing the interaction of *S. pneumoniae* and other respiratory pathogens using different approaches. The first study assessed the importance of bacterial co-infection in the 1918 pandemics by performing a systematic review of antemortem cultures from normally sterile sites and a meta-analysis of bacterial vaccine study to assess the importance of bacterial co-infection in the 1918 pandemics. The second study used a modeling approach investigating interaction of *S. pneumoniae* and influenza virus in an influenza pandemic with the same virulence characteristics as the 1918 virus. The third part assessed the interaction of three common respiratory bacteria *S. pneumoniae, H. influenzae,* and *S. aureus* in the nasopharynx using data from a longitudinal study in Peru. The subsequent four chapters describe these studies in the format of manuscripts for publication in peer-reviewed journals.

3.2. Objectives of the dissertation

Study 1: Evaluation of the importance of bacterial co-infection in the 1918 influenza pandemic

Study 1-1: Determine if the isolation rates of bacteria from antemortem cultures from normally sterile sites was related to disease severity (classified as pure influenza without pneumonia, influenza with pneumonia, influenza with subsequent fetal outcome) in the 1918 influenza pandemic through a systematic review.

Study 1-2: Determine if people who received bacterial vaccines and developed

influenza had statistically significant lower attack rates of pneumonia and lower case-fatality rates than unvaccinated influenza patients during the 1918 pandemic through a meta-analysis.

Study 2. Modeling the interaction of influenza virus and Streptococcus pneumoniae in future 1918-like influenza pandemics

Develop a Susceptible – Infectious – Recovered (SIR) model to

a. Determine if prevalence of pneumococcal colonization in the population influences the incidence of secondary pneumonia in a future 1918-like influenza pandemic.
b. Determine if antibiotic treatment for patients with secondary pneumococcal pneumonia can reduce the incidence of secondary pneumonia and mortality in a future 1918-like influenza pandemic.
c. Determine if antibiotic prophylaxis for patients with symptomatic influenza and antibiotic treatment for pneumonia patients can reduce the incidence of secondary pneumonia and mortality in a future 1918-like influenza pandemic

Study 3. The nasopharyngeal interactions of *Streptococcus pneumoniae, Staphylococcus aureus,* and *Haemophilus influenzae*

a. Determine if colonization status (presence/absence) of one of the three bacteria is associated with colonization status of the other two bacteria in the nasopharynx.
b. Determine if the density of one of the three bacteria is correlated with the density of the other two bacteria in the nasopharynx.

Chapter 4 – Study #1-1

Evidence from Ante-mortem Cultures for a Major Role of the Pneumococcus and Other Bacterial Pathogens in Mortality during the 1918 Influenza Pandemic

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ABSTRACT:

The 1918 pandemic caused an estimated 50 – 100 million deaths worldwide. We reviewed published ante-mortem cultures from normally sterile sites of 1918 patients to understand of role, if any, of secondary bacterial infections. Bacteria were rarely found in blood from influenza patients without pneumonia (1/409, 0.2%), but were commonly isolated from pneumonia cases (371/2365, 15.7%) and from living pneumonia patients with a subsequently fatal outcome (18/45, 40%). In addition, bacteria were recovered in pleural effusions or lung punctures from 227 of 285 pneumonia cases (79.6%). *Streptococcus pneumoniae* was found in 73.9% and 65.6% of the positive cultures from blood and lung, respectively. Secondary bacterial infections, especially pneumococcal infections, appear to have been a major contributor to mortality in the 1918 pandemic. The current lower prevalence of pneumococcal colonization, vaccination of children with pneumococcal conjugate vaccine (PCV), and widespread antibiotic use may have reduced the potential severity of a 1918-like pandemic in developed countries. A significant burden of pneumonia – related pandemic influenza mortality could still occur during the current H1N1 pandemic in developing countries with high prevalences of pneumococcal carriage, and with limited PCV and antibiotic access.

INTRODUCTION

The 1918 influenza pandemic was the most catastrophic infectious event in history with an estimated 50 – 100 million deaths worldwide.[1] Understanding the factors that led to such mortality may reduce mortality from the currently evolving novel H1N1 influenza pandemic and future pandemics. Current pandemic plans focus on stockpiling of antivirals and developing influenza vaccines against novel strains, reflecting the possibility that a highly virulent influenza virus led directly to most deaths in the 1918 pandemic by causing a primary viral pneumonia and acute respiratory distress syndrome.[2-6] The evidence to support the belief that deaths in 1918 resulted mainly from severe viral pneumonia appears to come from anecdotal stories of fulminant cases dying within hours after influenza onset,[7] and from animal studies showing that the reconstructed 1918 influenza virus can induce aberrant innate immune responses associated with extensive lung damage.[8-11]

There is however growing epidemiologic, clinical, and pathologic evidence suggesting that the majority of deaths in the 1918 pandemic resulted directly from secondary bacterial infections. Strikingly fulminant cases of death within hours represent only a small minority of the fatal cases.[12, 13] Brundage and Shanks recently summarized eight epidemiologic and clinical characteristics of this pandemic and hypothesized that sequential infection of influenza and common respiratory bacteria caused the highly fatal pneumonias in most victims.[13] The time course to death is also consistent with that of contemporary studies on pneumococcal pneumonia deaths.[12] Morens *et al* examined the recut lung tissue specimens from 58 influenza fatalities during the 1918 pandemic and found convincing histologic evidence of severe acute bacterial pneumonia in nearly all cases.[14] They also reviewed the published

post-mortem data, revealing that 92.7% of post-mortem lung cultures were positive for bacteria.[14]

As cultures during autopsy may be subject to contamination or post-mortem overgrowth, ante-mortem cultures from normally sterile sites are of particular value in establishing a bacterial etiology for pneumonia. We therefore reviewed published studies reporting ante-mortem cultures from sterile sites of patients during the 1918 pandemic to further understand the role of secondary bacterial infections.

METHODS

We identified papers from an archive at the National Institutes of Health, which includes thousands of reports in several major languages regarding the epidemiology, pathology and bacteriological findings from the 1918 pandemic.[14] All reports of ante-mortem cultures from blood, pleural effusions and lung punctures from influenza patients were sought and extracted from this archive. We stratified blood culture results according to the disease status at the time that samples were taken: uncomplicated influenza (without pneumonia); influenza cases with pneumonia; and influenza cases with pneumonia and subsequent fatal outcome.

Apparently-contaminated cultures were occasionally identified (e.g. non-hemolytic streptococci, *Streptococcus viridians* or *Staphylococcus albus*) and were counted as sterile. We excluded reports that did not separate influenza cases without pneumonia from pneumonia cases, as well as reports in which less than ten blood cultures were collected, or which did not provide actual numbers of cases studied.

RESULTS

We identified 60 reports of ante-mortem blood cultures from the 1918 pandemic. Eighteen studies that did not separate uncomplicated influenza patients from influenza – associated pneumonia patients, and eleven studies that examined less than ten cases, were excluded. Two further reports had been doubly – published. We therefore analyzed 29 studies of blood cultures obtained from 2774 patients. In addition, nine studies of ante-mortem cultures from lung and pleural cavities were identified; two were excluded because the results included patients both before and after clinical evidence of pneumonia, leaving seven studies of 285 patients.

Antemortem Blood Cultures

Uncomplicated influenza cases. Ten studies described blood cultures from 409 influenza cases without prevalent pneumonia.[15-24] Only one case (0.2%) yielded a pathogen; this case later developed streptococcal pneumonia and died.[15]

Influenza cases with pneumonia. Twenty four studies of ante-mortem blood cultures taken from 2365 patients with influenza – associated pneumonia were identified.[15, 16, 20, 23-45] The percentage of positive cultures in the 24 studies varied widely, from 1.6% to 50% (Table 4.1). Overall, 371 of 2365 blood cultures (15.7%) yielded bacteria. All but two of the 371 positive cultures were pure cultures.

Influenza cases with pneumonia and subsequent fatal outcome. Three of the 24 studies cited above reported the results of ante-mortem blood cultures from 45 pneumonia cases who later died; eighteen (40%) of them had positive ante-mortem blood cultures.[24, 28, 35]

Case fatality among bacteremic and non-bacteremic pneumonia patients. Four of the 24 studies reported the subsequent case fatality. Among 117 bacteremic pneumonia patients mortality was 69.2% (range 26.5% - 92.3%),[26, 29, 37, 38] compared to 18.2% mortality among 302 non-bacteremic pneumonia patients.[29, 37, 38]

Ante-mortem Cultures from Pleural Effusions and Lung Punctures

We identified seven reports examining 285 cases with influenza – associated pneumonia from whom ante-mortem cultures from pleural effusions or lung punctures had been taken (Table 4.2).[26, 30, 34, 38, 44-46] Bacteria were cultured from 227 overall (79.6%, range 56.7% - 100%); more than one pathogen was identified in 10.6% of these.

The Role of the Pneumococcus in the 1918 Pandemic

Streptococcus pneumoniae was the organism isolated most often from sterile sites. Of 371 positive blood cultures from pneumonia patients in the 24 studies (Table 4.1), 274 (73.9%) grew pneumococci, 81 (21.8%) grew hemolytic streptococci (probably *Streptococcus pyogenes*), 4 (1.1%) grew *Staphylococcus aureus*, and 14 (3.8%) grew other bacteria. The pneumococcus was the predominant bacterium in 16 of the 24 studies, often to the exclusion of all other organisms. Summarizing 227 positive cultures of pleural effusions and lung punctures from the seven reports (Table 2), 149 (65.6%) yielded pneumococci, 86 (37.9%) yielded hemolytic streptococci, and only one (0.4%) yielded *Staphylococcus aureus*.

The serotype distribution of the pneumococci identified from bacteremic pneumonia patients in the 1918 pandemic was very different from that seen before the pandemic.

Serotype was determined for 142 pneumococcal isolates from 11 of the 24 studies of blood cultures from pneumonia patients. Types I, II, III, and group IV pneumococci (which included atypical type II and all pneumococci of types 4 – 91 using modern nomenclature) were found in 12.7%, 25.3%, 12.0%, and 50.0%, respectively.[20, 23-26, 29, 34, 36, 37, 41, 42] In comparison, forty-six blood cultures of pneumonia patients from two reports published just prior to the 1918 pandemic showed 54.3% type I, 21.7% type II, 8.7% type III, and only 15.2% group IV.[47, 48]

DISCUSSION

Our review of ante-mortem bacteriologic findings during the 1918 pandemic suggests that secondary bacterial infections, especially pneumococcal infections, played an important role in the pathogenesis of influenza – associated pneumonia and death in this pandemic. Bacteria were rarely found in the blood of influenza patients without pneumonia (0.2%) but were commonly found in the blood of pneumonia patients (15.7%), in subsequently fatal cases (40%), and in the lungs of pneumonia cases (79.6%). The blood culture results were thus highly associated with severe pneumonia and mortality. Positive blood cultures, however, are an insensitive, if highly specific diagnostic modality to identify bacterial pneumonia. In the modern era, blood cultures are reported to be positive in 4% to 18% of adult patients hospitalized with community-acquired pneumonia.[49] A clinical trial of pneumococcal conjugate vaccine (PCV) suggests that only 2.6% of children with pneumococcal pneumonia had positive blood cultures.[50] Given the insensitivity of blood cultures and the high percentage of positive cultures from the lungs, these data add considerable biologic plausibility to the post-mortem bacteriology and pathology studies[14] that the majority of pneumonias and deaths in the 1918 pandemic resulted from secondary bacterial

infections.

The percentage of positive blood cultures in pneumonia cases varied greatly in these studies (Table 1). Although the reports provide little information about their microbiological methods, we believe that the variability is unlikely due to microbiologic technique. Culture techniques for pneumococci, hemolytic streptococci and staphylococci were standardized in textbooks before 1918.[51] The differences among studies were most likely due to the time at which the blood samples were collected. Positive blood cultures were usually taken from sicker patients or shortly before death.[29, 31] For example, one study showed that eight of 11 blood cultures taken from patients who died <24 hours yielded bacteria (72.7%), while only one of 6 cultures taken >24 hours before death was positive (16.7%).[28] In another study seven of 11 positive blood cultures were taken just prior to death.[16]

Although the majority of the studies showed a high prevalence of bacteremia among pneumonia cases, four reported a low percentage (<10%; Table 1), probably because these cultures were taken early in the disease course or from milder cases. At Camp Custer, where only 2.2% of ante-mortem blood cultures from pneumonia patients were positive, 228 (91.2%) of 250 post-mortem lung cultures and 165 (65.7%) of 251 heart blood cultures yielded bacteria, of which 78 (34.2%) and 77 (46.7%) were pneumococci, respectively.[16] At Camp Cody, where only 1.6% of ante-mortem blood cultures were positive, bacteria were recovered at autopsy from the lung (100%) and heart blood (71%) of the 14 fatal cases examined.[23, 45] In that camp, attempts were made to detect influenza and pneumonia cases as early as possible to keep them apart from well soldiers. Moreover, pneumonia diagnoses were made based on non-specific

auscultatory findings and it is likely that the low rate of blood culture positivity in that and some other series was due in part to "over-diagnosis" of pneumonia in patients with uncomplicated influenza.[23]

Rates of positive blood and lung cultures did not appear to differ between civilian and military pneumonia patients (Tables 1 & 2). In the US military camps, the 1918 attack rate of influenza was 21%; of these, 17% developed pneumonia, with a mortality of 34%.[52] The case fatality among pneumonia cases in the civilian population was similar (30%),[53] consistent with the similar culture results found. The civilian population also had comparable influenza attack rates; however, the attack rate of secondary pneumonia was higher in military camps.[53] This observation suggests that the influenza virus was equally contagious in military camps and general populations, but that bacterial transmission was more intensive in military camps, resulting in higher incidences of pneumonia.

Animal studies during the 1930s and 1940s revealed that influenza virus in combination with respiratory bacteria, such as *Haemophilus influenzae*[54, 55] and hemolytic streptococci,[56-59] can act synergistically to increase disease severity and cause death. Recent studies in mice have shown not only the biologic plausibility of this synergistic lethality, but also that the 1918 influenza virus may have had a particular propensity to synergize with pneumococci.[60-62]

Our review suggests that pneumococci were the most important cause of influenza – associated pneumonia and death, and that *Staphylococcus aureus* was less commonly found in the 1918 pandemic. In the subsequent pandemics of 1957-1958 and

1968-1969, secondary pneumonias and overall mortality were much lower,[63, 64] but pneumococci were still the predominant pathogen isolated from ante-mortem blood or sputum cultures of pneumonia cases.[65-68] *Staphylococcus aureus*, however, was the predominant organism isolated from post-mortem lung cultures in the 1957-1958 pandemic,[69, 70] probably because this organism was associated with severe pneumonia and high prevalence of resistance to commonly prescribed antibiotics.[65] Moreover, a somewhat lower percentage of positive post-mortem lung cultures were found in the 1957-58 pandemic than in the 1918 pandemic (80% vs 92.7%), possibly due to widespread antibiotic use.[14, 70] Pneumococci were the major causes of pneumonia in all three pandemics, but the mortality rates and causes of death were different. Although decreasing viral virulence could have contributed to this change, the availability of antibiotic treatment was, in our view, also of importance.

Pneumococci could only be classified into 4 – 5 types in the 1910s, while currently 91 serotypes have been identified. By 1918 group IV pneumococci were considered common but less invasive colonizers in healthy individuals, while type I pneumococci – then as now – were more commonly found in blood.[71-73] The high prevalence of group IV pneumococcal bacteremia during the 1918 pandemic suggests that the 1918 influenza virus increased host susceptibility to secondary infections with less virulent pneumococci.

Our review has several limitations. First, the archive may have missed some studies; however, to our knowledge, there is no more comprehensive archive containing bacteriologic data from the 1918 pandemic. Second, most studies provided little information about the microbiologic techniques, diagnostic criteria for pneumonia, or

selection criteria of the cases, making it difficult to perform quality assessment and assure comparability of the studies. Third, the methods of bacterial identification and classification in 1918 were somewhat different from those used today. For example, some type III pneumococci might have been classified as *Streptococcus mucosus.*[74] Fourth, the use of blood cultures to identify bacterial infections is insensitive, and undoubtedly underestimates the role of secondary bacterial infections in pneumonia and mortality. It is also possible that the role of bacteria such as *Haemophilus influenzae*, which was not easily cultured in 1918 and is generally less likely to cause bacteremia, may have been underestimated. Finally, the studies we examined mainly came from military camps and tertiary hospitals, and may not be generalizable.

If pneumococcus were the major cause of death during the 1918 pandemic, what the public health implications are for a 21st century pandemic? Before the 1918 pandemic, the prevalence of pneumococcal colonization in healthy adults was estimated to be around 40%.[71, 75, 76] This is much higher than that in developed countries today (usually <10%),[77-79] though a similar high prevalence may still be found in developing countries.[80] The widespread use of PCV in infants in developed countries has reduced the incidence of invasive pneumococcal disease in children and also in adults due to herd immunity, though this vaccine only contains 7 of the 91 serotypes.[81] Moreover, a clinical trial of PCV has shown that vaccinated children had a 45% reduction in seasonal influenza-related hospitalization for pneumonia.[82]

Given the above facts and the ready availability of antibiotics in developed countries, the appearance of a pandemic influenza virus as pathogenic as that of 1918 would be expected to result in many fewer deaths than occurred in 1918 – 1919. With antivirals

and the possible availability of a specific influenza vaccine within a few months after the identification of the pandemic strain, incidence of secondary bacterial pneumonias and deaths could be further decreased. Nevertheless, a higher incidence of pneumonia and mortality is possible in developing countries due to higher pneumococcal carriage and more limited access to PCV, antibiotics, antivirals and influenza vaccines.

In the current evolving H1N1 pandemic, there has to date been only limited evidence of secondary bacterial infection as a major cause of hospitalization,[83] but data from fatal cases remain to be reported. The apparently lower pathogenicity of the virus and the widespread use of antivirals, PCV and antibiotics, as well as the summer (Northern Hemisphere) epidemic at a time when pneumococcal carriage is lower,[84] may have contributed to the low rate of pneumonia and mortality to date. In addition, antibiotic administration decreases the sensitivity of routine culture methods to detect secondary bacterial infections. In countries experiencing disease in winter, with higher rates of pneumococcal carriage and little access to antivirals and influenza vaccines, early antibiotic therapy to prevent secondary bacterial infections might be considered in influenza – infected people, particularly for those at high risk of pneumonia, such as the elderly and persons with underlying chronic diseases.

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Table 4.1: Blood cultures among pneumonia patients in the 1918 pandemic.

Place	Population	No. cases	No. positive B/C	% positive B/C	No. positive pneumococci	No. positive hemolytic streptococci	No. positive *S. aureus*	No. other bacteria or undetermined
Camp Grant [25, 44]	Military	90	45	50	45	0	0	0
Camp Beauregard [26]	Military	111	53	47.7	50	3	0	0
Puget Sound Navy Yard [27]	Military	52	24	46.2	0	23	0	1
Amsterdam [28]	Civilian	21	9	42.9	3	3	0	3[a]
Calcutta, India [15]	Civilian	76	30	39.5	14	12	2	2[b]
Flanders and France [29]	Military	33	13	39.4	13	0	0	0
Liverpool [30]	Military	30	11	36.7	9	3	0	0
Fort Riley [31]	Military	17	6	35.3	3	3	0	0
UK Navy [32]	Military	20	7	35.0	4	3[c]	0	0
A Scottish Hospital [33]	Civilian	15	5	33.3	5	0	0	0
St. Louis [20]	Civilian	41	10	24.4	4	2	0	4
John Hopkins Hospital [24]	Civilian	25	6	24.0	5	1	0	0
Camp Devens [34]	Military	118	28	23.7	27	1	0	0
Minneapolis [35]	Civilian	15	3	20	3	0	0	0
DC Navy Hospital [36]	Military	40	8	20	8	0	0	0
Camp MacArthur [37]	Military	233	34	14.6	31	0	2[d]	2
Fort Sam Houston [38]	Civilian	111	16	14.4	1	13	0	2
Camp Travis [39]	Military	178	21	11.8	21	0	0	0
US Troops in England [40]	Military	126	14	11.1	12	2	0	0
Sydney [41]	Civilian	19	2	10.5	1	1[c]	0	0
Camp Sherman [42]	Military	100	6	6	6	0	0	0
Camp Lee and Camp Dix [43]	Military	136	5	3.7	5	0	0	0
Camp Custer [16]	Military	510	11	2.2	1	10	0	0
Camp Cody [23, 45]	Military	248	4	1.6	3	1	0	0
Total		2365	371	15.7	274	81	4	14

[a] Not speciated, pneumococci or hemolytic streptococci.
[b] Two capsulated Gram-negative cocci, possibly meningococci. Ten cases of non-hemolytic streptococci and 4 cases of unknown bacteria (Gram-positive motile bacillus) were considered contaminated.
[c] Hemolytic property not mentioned in the reports.
[d] Not known whether these were *S. aureus* or *S. albus (epidermidis)*

Table 4.2: Cultures from pleural effusions or lung puncture among pneumonia patients in the 1918 pandemic.

Place	Population	No. B/C taken	No. positive B/C	% positive B/C	No. positive pneumococci	No. positive hemolytic streptococci	No. positive *S. aureus*	No. other bacteria or undetermined
Liverpool [30]	Military	17	17	**100**	13	11	0	0
Chicago [46]	Civilian	8	8	**100**	5	3	0	1
Camp Grant [44]	Military	74	73	**98.6**	51	27	0	0
Camp Deven [34]	Military	44	36	**81.8**	29	5	0	8
Camp Beauregard [26]	Military	29	22	**75.9**	17	3	0	2
Fort Sam Houston [38]	Civilian	53*	37	**69.8**	4	28	1	4
Camp Cody [45]	Military	60	34	**56.7**	30	9	0	0
Total		285	227	**79.6**	149	86	1	15

* This study examined lung punctures from 20 cases and pleural effusions from 33 cases. The other studies only reported cultures from pleural effusions.

Chapter 5 – Study #1-2

Efficacy of whole-cell killed bacterial vaccines in preventing pneumonia and death during the 1918 influenza pandemic

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Abstract

Background. Most deaths in the 1918 influenza pandemic were caused by secondary bacterial pneumonia.

Methods. We performed a systematic review and re-analysis of studies of bacterial vaccine efficacy in preventing pneumonia and mortality among influenza patients during the 1918 pandemic.

Results. A meta-analysis of six civilian studies of mixed killed bacterial vaccines containing pneumococci identified significant heterogeneity among studies and estimated vaccine efficacy (VE) at 34% (95% CI 19–47%) in preventing pneumonia and 42% (18–59%) in reducing case-fatality rates among influenza patients using random effects models. The pooled VE from three military studies was 59% (95% CI 43–70%) for pneumonia and 70% (95% CI 50% – 82%) for case fatality using fixed effect models, respectively. Military studies showed less heterogeneity and may provide more accurate results than civilian studies, given the potential biases in the included studies. One military study using hemolytic streptococci also suggested significant protection.

Discussion. This re-analysis concludes that despite significant methodological problems, the systematic biases in these studies do not exclude the possibility that whole-cell inactivated pneumococcal vaccines may confer cross-protection to multiple pneumococcal serotypes and that bacterial vaccines may play a role in prevention of influenza-associated pneumonia.

Key words: (3 – 10 key words)

Influenza

Pandemic

Streptococcus pneumoniae

Vaccine

Bacteria

Co-infection

Secondary bacterial pneumonia

Introduction

The 1918 influenza pandemic caused an estimated 20–100 million deaths worldwide [1]. There is growing epidemiologic, clinical, and pathologic evidence that the majority of deaths in this and subsequent pandemics resulted directly from secondary bacterial pneumonia [2-5]. In the 1918 pandemic *Streptococcus pneumoniae* was the predominant organism isolated from antemortem cultures of normally sterile sites of influenza-associated pneumonia patients, followed by hemolytic streptococci presumably representing *Streptococcus pyogenes* [4, 5].

The etiology of influenza was unknown at the time of the 1918 pandemic. Many contemporaneous investigators erroneously believed that bacteria, in particular *Bacillus influenzae* (Pfeiffer's bacillus, now known as *Haemophilus influenzae*) was the cause of influenza [6]. It was also however generally believed that most 1918 pandemic influenza deaths resulted from secondary bacterial pneumonia following primary influenza infections of whatever cause [2]. In attempts to prevent the primary disease of influenza, to reduce pneumonia and mortality, and to investigate the etiology of influenza, many bacterial vaccines were produced, tested, and administered during the 1918 pandemic.

Here we review studies of whole-cell bacterial vaccines administered to healthy subjects during the 1918 pandemic to examine their efficacy in preventing influenza-associated pneumonia and mortality.

An important concern about such a review is that by today's standards, the scientific quality of 1918 vaccine studies was low due to such methodological issues as lack of subject randomization. Moreover, while most vaccinations were given during the declining phase of the pandemic (fall-winter 1918-1919), the incidences of influenza, influenza-associated pneumonia, and deaths in vaccinated individuals,

were usually compared to the same outcomes in unvaccinated individuals from the beginning of the epidemic [6, 7], introducing unequal observation periods more favorable to vaccinated individuals. In addition, vaccinated individuals might come from select populations with reduced exposure or susceptibility to influenza because they had not had influenza between the appearance of the pandemic and the start of vaccination. Not fully appreciating such potential design flaws, investigators studying bacterial vaccines often believed they had demonstrated a reduction in the incidence of influenza, which is not consistent with our understanding of influenza etiology.

We reasoned that any true effect of bacterial vaccines on influenza disease might more plausibly result from reduced attack rates of secondary bacterial pneumonias and consequent reduced case-fatality rates among influenza-infected patients. To examine this possibility while addressing methodological flaws of the original studies, we re-analyzed published data asking whether vaccinated patients who developed influenza had lower attack rates of pneumonia or lower case-fatality rates than unvaccinated influenza patients. This approach should diminish bias caused by unequal observation periods because these measures were less likely to be influenced by changing influenza incidence during the progress of the pandemic. In addition, the attack rate of pneumonia and case-fatality rates among influenza patients seems to be higher in the later phase of influenza epidemics [8-10]. Therefore, this approach may result in more conservative estimates of vaccine efficacy because the vaccinated people were more likely to be from the later phase of the 1918 pandemic.

Methods

Search strategy and criteria

In an effort to obtain all relevant publications reporting bacterial vaccine

studies in the 1918 pandemic, a literature search was performed on the Journal Storage database (JSTOR) using the search terms "influenza or flu," "vaccine or vaccination or inoculation" and "year: 1918 to 1920" in the full text without language restriction. We also manually searched two bibliographic sources — the *Index Medicus* and the *Index-Catalogue of the Library of the Surgeon – General's Office* — for relevant papers in any language between 1918 and 1920. In addition, we examined all papers from an archive at the National Institute of Allergy and Infectious Diseases, National Institutes of Health (http://www3.niaid.nih.gov/topics/Flu/1918/bibliography.htm) [3]. The archive was originally developed to identify publications containing information on influenza pathology and bacteriology in the 1918 pandemic, but was expanded to contain other topics. We examined all retrieved articles to identify additional papers.

Selection criteria and data extraction

Original reports of prophylactic administration of bacterial vaccines to humans during the autumn 1918 or winter 1918-1919 pandemic waves were eligible for inclusion. We then searched for studies in which case-fatality rates or attack rates of pneumonia among both vaccinated and unvaccinated influenza patients could be determined. Vaccinated influenza patients were defined as clinically-diagnosed influenza patients who had received at least one dose of a bacterial vaccine at any time before the onset of influenza. We excluded reports which did not provide exact denominators (the number of vaccinated and unvaccinated influenza cases) or in which one or both of the vaccine exposure denominators was less than ten. When multiple publications reported results from the same study population, only results from the most recent publication were included. Because these early papers did not

provide much details, we assessed the quality of study using four criteria: (1) whether vaccinees were randomized, (2) whether vaccination was completed before the occurrence of the first influenza patients in the facility, (3) whether the vaccinated and unvaccinated group were from the same population, and (4) we considered studies to be of better quality if the bacterial vaccine given was not reported to reduce the incidence of influenza among the vaccinated compared to unvaccinated subjects.

Statistical methods

Unadjusted risk ratios (RRs) comparing case-fatality rates and pneumonia attack rates in vaccinated and unvaccinated influenza patients were calculated with 95% confidence intervals for each included study. When there were no pneumonia cases or deaths recorded for a study group, a value of 0.5 was assigned. Vaccine efficacy (VE) was calculated as 1– RR.

We stratified the studies according to the vaccine formula and study population (civilian or military). Meta-analysis was performed on studies of bacterial vaccines containing pneumococci. An estimate of heterogeneity across studies was assessed using Q statistics and I^2 statistics; a p-value below 0.10 (Q statistic) or an I^2 value greater than 50% was considered significant [11]. When significant heterogeneity was found, pooled RR estimates and 95% confidence intervals were derived using a random effects model; otherwise, a fixed effect model with Mantel-Haenszel weighting was used. Publication bias was assessed by using funnel plots [11]. We explored the sensitivity of the meta-analysis results by (1) examining whether the results were strongly influenced by excluding each included study one at a time, and (2) using the "trim-and-fill" method to adjust for potential publication bias [12]. Analyses were performed using free MIX 1.7 software available on

http://www.mix-for-meta-analysis.info/ [13, 14].

Results

Study selection

We identified and retrieved full texts of 485 publications for assessment. Figure 1 summarizes the study selection process. Thirteen studies were included in the final analysis.

Characteristics and quality of included studies

Information on the vaccines in the thirteen studies is shown in Table 5.1. Eight studies reported mixed inactivated vaccines containing multiple serotypes of *Streptococcus pneumoniae* in addition to other bacteria, such as *B. influenzae*, hemolytic streptococci, or *Staphylococcus aureus* [15-22]. Four studies utilized a vaccine containing multiple strains of *B. influenzae* [23-26] and the remaining study used a vaccine containing multiple strains of hemolytic streptococci [27]. The strains of bacteria used in the vaccines were usually obtained from cases during local influenza epidemics. The vaccines were whole-cell bacterial vaccines inactivated by heat, tricresol or chloroform. The amount of each organism and inoculation schedules differed between studies.

The characteristics of the thirteen studies are shown in Table 5.2. Seven studies were from civilian and three from military populations. The Cadman study reported military and civilian data separately [16]. The Minaker study reported that vaccinated individuals were mainly from the military while unvaccinated individuals were from the civilian population [21]; this study was regarded as a civilian study in our analyses.

None were double-blinded randomized trials. The quality of the McCoy and Hilton studies was highest because vaccinated individuals were assigned in a random fashion and the vaccination completed before the outbreaks appeared in the facilities where the vaccination was performed [20, 24]. The Minaker study was of lowest quality because vaccinated and unvaccinated persons were from different populations [21]. For the rest of the studies, vaccinated and unvaccinated subjects were from the same military or civilian populations, but it was not possible to fully evaluate their comparability due to insufficient information on potential confounders such as age, gender, and health status, as well as how vaccinated individuals were chosen.

Table 5.3 shows the incidence of influenza among vaccinated and unvaccinated subjects, as reported in the original analyses of all the studies except for the Cherry study which also used influenza patients as the denominator in the analysis [17]. The incidence of influenza among unvaccinated subjects varied from 3.5–38.5%, probably reflecting, among other factors, differences in case identification. According to the US house-to-house survey, approximately 28% of population had an influenza attack in the 1918 pandemic [28]. Three studies reporting an influenza incidence of less than 10% probably used hospital admission records for case identification, while studies reporting influenza incidence close to 28% may have included influenza outpatients.

Except for two studies with random allocation [20, 24] and one small study [26], the included studies reported lower incidence of influenza in vaccinated subjects, presumably because vaccination usually began after the epidemic had occurred. We re-analyzed the original data including only diagnosed influenza patients in the denominators to compare attack rates of influenza-associated pneumonia and case-fatality among vaccinated and unvaccinated influenza patients.

Effect of mixed bacterial vaccines containing pneumococci on the attack rate of pneumonia

RR estimates for the comparison of pneumonia attack rates between vaccinated and unvaccinated influenza patients ranged from 0.46 to 1.17 in five civilian studies (Figure 2). Three of the five studies showed significant vaccine protection against pneumonia, but the McCoy study with the highest quality suggested no protective effect. There was heterogeneity among the civilian studies ($P < 0.0001$ for Q statistic; $I^2 = 86.23\%$). The random effects estimate of pooled RRs was 0.66 (95% CI 0.53–0.81) — a VE of 34% (95% CI 19–47%). Combined RRs changed most by excluding the Cherry study [25]; after excluding this study, no heterogeneity was indicated and pooled VE using a fixed effects model was 31% (95% CI 26–35%). The funnel plot suggested potential publication bias and the trim-and-fill adjusted VE was 40% (95% CI 26–51%) for the civilian studies.

RR estimates from three military studies ranged from 0.35 to 0.55, two of which were statistically significant (Figure 2). There was no heterogeneity for the military studies ($P = 0.6269$ for Q statistic; $I^2 = 0\%$). The pooled VE using a fixed effect model was 59% (95% CI 43–70%). After excluding the Cadham study, the pooled VE was 57% (95% CI 37–70%). The trim-and-fill adjusted VEs was 60% (95% CI 46–70%).

Effect of mixed bacterial vaccines containing pneumococci on case-fatality rates

Four of six civilian studies showed a significant protective effect of the bacterial vaccines on reducing influenza case-fatality rates, but the best-quality McCoy study did not suggest any vaccine effect (Figure 3). There was significant

heterogeneity (P < 0.0001 for *Q* statistic; I^2 = 81.47%). The random effects pooled RR among civilian studies was 0.58 (95% CI 0.41–0.82), or 42% (95% CI 18–59%) VE. After excluding the Cherry study, which had the strongest influence on meta-analysis results, no heterogeneity was indicated and the fixed effect VE estimate was 34% (95% CI 27–41%). The funnel plot did not suggest publication bias.

RR estimates from three military studies ranged from 0.19 to 0.45, all statistically significant (Figure 3). There was no heterogeneity for the military studies (P= 0.3595 for *Q* statistic; I^2 = 2.25%). The fixed effect estimate of VE was 70% (95% CI 50% – 82%). After excluding the Leishman study, the combined efficacy was 65% (95% CI 41–79%). The trim-and-fill efficacy adjusted for potential publication bias was 62% (95% CI 37–77%).

Effect of bacterial vaccines without pneumococci

Four civilian studies utilized bacterial vaccines containing pure *B. influenzae* (Table 5.4). The Hilton study with random allocation of vaccination did not found a vaccine effect in reducing case fatality, and so did other two studies [25, 26]. However, the remaining Duval study suggested that vaccine efficacy for reducing the attack rate of pneumonia was 94% (p<0.0001) [23]. Hemolytic streptococci were the main cause of influenza-associated pneumonia in the Ely study, which reported use of a vaccine containing only this pathogen [27]; none of the 144 vaccinated influenza patients died and the estimated RR was 0.05 (p<0.0001), corresponding to a VE of 95% (95% CI 19–100%).

Discussion

Strengths and Limitations

The quality of vaccine studies in 1918-1919 was lower than studies

conducted today because accepted modern approaches to study design and evaluation were unknown or not well recognized in 1918. In addition, due to the scope of the 1918 pandemic and the exigency of war, medical personnel were forced to work under a great strain, so it was difficult to obtain complete data and perform good trials at that time [16].

Misclassification of influenza or pneumonia could occur in the vaccine studies we examined because diagnosis was based largely on physical examination using unstandardized diagnostic criteria. Vaccinated people suffering from constitutional adverse reactions to the vaccine might be misdiagnosed as influenza cases though these reactions usually appeared early and were of short duration [23]. Influenza cases diagnosed late in the pandemic may reflect respiratory illness from less virulent viral infections when influenza activity decreased, potentially introducing differential misclassification because vaccinated people were usually from this phase of the pandemic. Chest X-rays were available at that time, but we do not know the extent to which chest X-rays were used in these studies to diagnose pneumonia. Since death is an outcome less susceptible to misclassification, analysis of case fatality should be less susceptible to bias. Except for two studies using random allocation, other studies failed to control for important confounders. Because of population homogeneity, better standardized diagnosis and case identification, military studies should potentially provide more valid estimates than civilian studies by controlling for factors that might influence pneumonia attack rates and case-fatality rates, such as age, health status and environmental exposure, as well as reducing misclassification.

Subject self-selection was another potential problem in the 1918 vaccine studies because vaccination was usually given voluntarily. However, the direction of potential “volunteer bias” is difficult to determine and might differ among studies.

One vaccine study conducted shortly after the pandemic found that high-risk individuals were more likely to be vaccinated [29], which would have resulted in bias toward the null. On the other hand, "healthy vaccinee bias" is well described in observational influenza vaccine studies today and could have played a role in the 1918 studies [30]. It is unlikely that vaccination self-selection based on health status occurred in military populations, since the military is fairly homogeneous and selected for excellent health.

Finally, while we sought to identify all existing papers, there may be additional studies that we did not find. However, we believe that missing reports would not have biased our results in a specific direction because most contemporaneous vaccine studies did not use influenza patients as the denominators in their analyses as we did. Although we only searched reports published until the end of 1920, we think that the time window we selected would have covered vaccine trials pertinent to the pandemic years because publication of clinical studies happened much faster then than it does now. The latest study included in our analysis was published in February, 1920 [19].

Despite these limitations, we believe that our method of analysis could remove biases caused by unequal observation periods and the subgroup analysis of military studies may be less susceptible to other sources of bias. The estimated VE of bacterial vaccines containing pneumococci for preventing case fatalities in the military studies (70%) may be the most accurate figure in our analyses because of less confounding, misclassification and self-selection.

We hesitate to interpret findings in civilian studies because residual biases could still be large in some civilian studies even using our method of analysis. Studies of *B. influenzae* provide a chance to examine this possibility because it was not an

important cause of secondary pneumonia in 1918 [4, 5]. Our analyses seemed to completely remove biases in the Barnes study [25] because no protective effect of *B. influenzae* vaccines was found using our method while the original analysis showed a significant protective effect. However, the Duval study still estimated a high VE of this vaccine in preventing pneumonia using our analyses [23]. This study reported a very high attack rate of pneumonia among unvaccinated influenza patients (32%), suggesting that the unvaccinated influenza patients in this study were a very special population and probably not a fair comparison group. This also reminds us the limitations of observational vaccine studies and we need to be cautious interpreting their results, because biases may not be completely removed even with good statistical analyses.

Biological plausibility

It has been suggested that most of the US Army training camps around 1918 experienced "colonization epidemics" with specific pathogenic bacteria, either pneumococci or hemolytic streptococci, which resulted in a huge number of pneumonia cases caused by these two bacteria during epidemics of measles (winter 1917-1918) and of influenza (fall 1918 and winter 1918-1919) [3, 31]. The effect of locally produced bacterial vaccines thus depended on the bacteria circulating locally. It is very likely that at least 70% of military deaths were caused by secondary pneumonia because soldiers were healthy adults unlikely to die due to deterioration of underlying medical conditions caused by influenza. In addition, one military study published in 1989 found that the pneumococcal carriage was 1% among healthy men entering military service as compared to 13% among healthy recruits already in service [32], suggesting higher colonization prevalence and higher transmission of the

pneumococcus in barracks. Such a high VE may be less plausible if these whole-cell vaccines only provided type-specific protection, due to the diversity of serotype distribution of pneumococci in 1918 [4]. Some of these vaccines included multiple pneumococcal strains known to be causing local epidemics and commonly isolated from pneumonia or fatal cases, but no systematic attempt could be made to identify and include strains beyond serotypes I – III as the serological tools to identify these strains were in their infancy. Recent animal studies also support the possibility that whole-cell pneumococcal vaccines induce cross-protective (i.e., more broad than serotype-specific) immunity [33, 34]. In contrast to the diversity of the pneumococcus in the 1918 pandemic, although only one small military study used a hemolytic streptococcal vaccine alone, if it represented the dominant strain of Group A streptococcus causing the disease in the camp at that time, a high level of efficacy may be biologically plausible. Epidemics caused by a single M-type Group A streptococcus have been shown in later military studies [35, 36].

While the possibility of unappreciated biases is important to consider and the best-quality McCoy study with a small sample size suggested no vaccine effect, the general consistency of the data indicating a protective effect for the two types of bacterial vaccines designed to prevent the now-accepted major causes of pneumonia and death in the 1918-1919 pandemic (pneumococci and hemolytic streptococci) are consistent with biologic effects [4, 5].

Implications

This review supports the idea that while secondarily-infecting bacteria played a major role in influenza-associated pneumonia and mortality in the 1918 pandemic, bacterial vaccines containing pneumococci could potentially reduce

influenza-associated pneumonias and deaths in modern pandemics. There are few contemporary studies that have evaluated bacterial vaccinations in seasonal or pandemic influenza. A double blind randomized trial of a 9-valent pneumococcal conjugate vaccine given to young infants had a 45% efficacy in reducing seasonal influenza-associated pneumonia [37].

Even with the current availability of antibiotics, autopsy series using modern molecular technique from the 2009-2010 H1N1 pandemic suggest that bacterial infections, particularly pneumococcal infections, were implicated in 29–55% of deaths [38-40]. The current H1N1 pandemic has led to a shift in the age distribution of case hospitalization, severe pneumonia, and death, from the expected elderly age groups to older children and young adults who have, in most other influenza pandemics and in seasonal influenza, been at low risk of influenza-associated complications [41, 42]. A recent study of this age group shows that the presence of the pneumococcus was strongly correlated with severe disease and death (odds ratio, 126) [43], consistent with the possibility that unexplained mortality in otherwise healthy young people in 1918 could also have been due to dual infections with influenza and pneumococci.

It is a challenge to review these old vaccine studies but we believe our method of analyses and the examination of bias have made these early data more interpretable. Although these analyses cannot provide conclusive evidence of the efficacy of whole-cell pneumococcal and group A streptococcal vaccines in preventing bacterial superinfections in influenza patients, we believe they do support further investigation of killed bacterial vaccines in the prevention of pneumococcal pneumonia, influenza-associated pneumonia, and mortality. The 1918 VE data presented here suggest to us the possibility that cheap whole-cell pneumococcal

vaccines eliciting cross-protection against multiple pneumococcal serotypes may be worthy of re-consideration.

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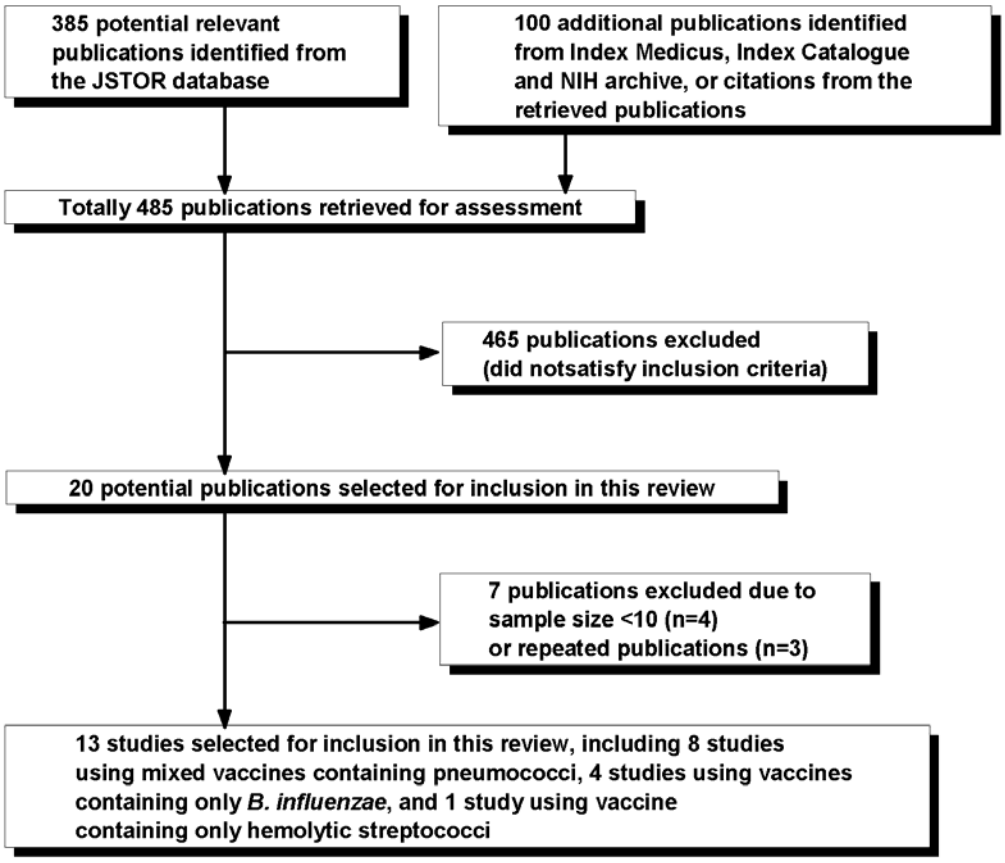

Figure 5.1: Selection of published studies of bacterial vaccines in the 1918 influenza pandemic.

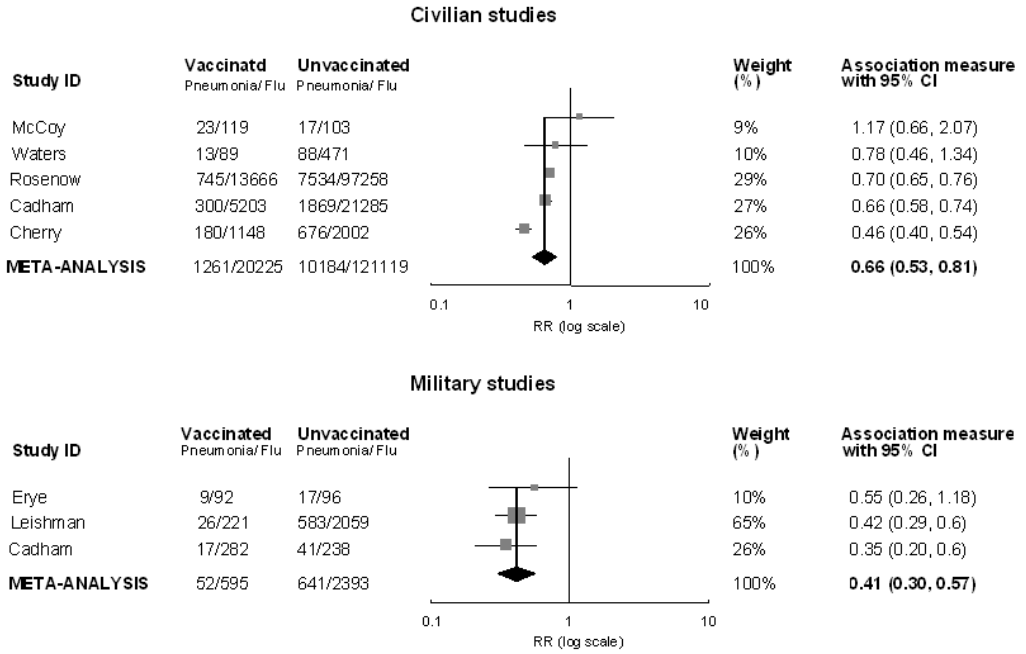

Figure 5.2: Random effects meta-analysis of eight RR estimates comparing attack rates of pneumonia among vaccinated and unvaccinated influenza patients in studies of bacterial vaccines containing pneumococci, stratified by study population (civilian or military). RR < 1 indicated that the vaccine was protective. Point estimates and 95% confidence intervals (CIs) are shown for each study and for pooled results. Data are plotted on a log base 10 scale.

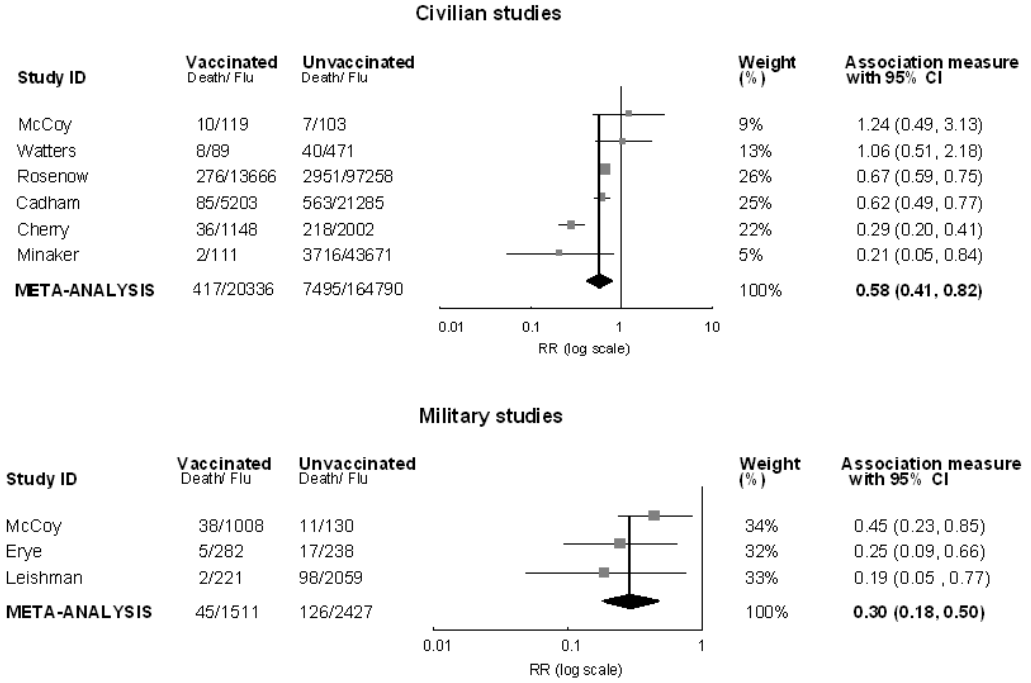

Figure 5.3: Random effects meta-analysis of nine RR estimates comparing case-fatality rates among vaccinated and unvaccinated influenza patients in studies of bacterial vaccines containing pneumococci, stratified by study population (civilian or military). RR < 1 indicated that the vaccine was protective. Point estimates and 95% confidence intervals (CIs) are shown for each study and for pooled results. Data are plotted on a log base 10 scale.

Table 5.1: Vaccine contents, dosages and preparation methods in the 12 included studies (may be in online appendix)

Study	Vaccine contents, organisms, millions/mL[a]	Dosage	Inactivation method	Sources of bacteria strains used in vaccine
Cadham [15]	Pneumococci (300 military, 600 civilian), streptococci (600 military, 300 civilian), *Bacillus influenzae* (400 for both groups)[b]	2 doses of 0.5 mL at 7-d interval	Heat	Streptococci were obtained from empyema, nasopharyngeal, blood, and postmortem lung cultures; strains of pneumococci were isolated from nasopharyngeal and sputum cultures; *B. influenzae* was isolated from nasopharyngeal cultures obtained from the first patients recognized as having typical cases of pandemic influenza in Winnipeg in October 1918. Strains of bacteria used in military studies were locally isolated; some civilian populations received vaccines containing strains obtained from E. C. Rosenow (Mayo Clinic, Rochester, MN).
Cherry [16]	Pneumococci (10, 50), streptococci (10, 50), *B. influenzae* (25, 125) *Moraxella catarrhalis* (25, 125), a gram-positive diplococcus other than the pneumococcus (10, 50)	2 doses of 1 mL at 7-d interval [17]	Tricresol	Pneumococci were not classified in Australia, but 6–16 strains were included in each batch of the vaccine; multiple strains were also used for other organisms. All included strains were isolated from patients with influenza during the epidemics in Australia and South Africa in late 1918 and early 1919; some were of postmortem origin [17].
Erye and Lewe [18]	Pneumococci (100, 200), streptococci (20, 100), *Staphylococcus aureus* (400, 1000), *B. influenzae* (20, 60), *M. catarrhalis* (50, 150), other *Bacillus* species (200, 400)	2 doses of 0.5 mL at 10-d interval	Not reported	Fresh strains of streptococci, pneumococci, and *B. influenzae* were obtained from patients with virulent infection (septicemic influenzal pneumonia) at a naval hospital and a transport ship arriving at a port in the United Kingdom.
Leishman [19]	Pneumococci (200), streptococci (80), *B. influenzae* (60)	2 doses of 0.5 and 1 mL at 10-d interval	Heat	Several strains and types of each organism, all isolated relatively freshly from case patients
McCoy et al [20]	Pneumococcal types I–IV (total, 3000), hemolytic streptococci (1000), *S. aureus* (500), *B. influenzae* (500)	3 doses of 0.5, 1, and 1.5 mL at 48-h intervals	Not reported	>2 strains of each organism; sources were not reported.
Minaker and Irvine [21]	Pneumococcal types I–III (total, 7000), hemolytic streptococci (100), *B. influenzae* (5000)	3 doses of 0.5, 0.8, and 1 mL at 3-d intervals	Heat	*B. influenzae* was obtained not locally but from the Rockefeller Institute; sources of the other bacteria were not reported.
Rosenow and Sturdivant [22]	Pneumococcal types I–III (total, 3000), hemolytic streptococci (2000), *S. aureus* (1000), pneumococcal type IV and allied green-producing diplostreptococci (4000)[c]	3 doses of 0.25, 0.5, and 0.75 mL at 7-d intervals	Heat	Authors stressed the importance of using freshly isolated strains because of the tendency of bacteria to lose virulent properties; they also stated that the composition of vaccine should be adjusted frequently to reflect changes in the bacterial strains in circulation.
Watters [23]	Pneumococci (400), hemolytic streptococci (400), *B. influenzae* (100), *M. catarrhalis* (400)	3 doses of 0.2, 0.3, and 0.4 mL at 3-d intervals	Not reported	Organisms isolated from lungs at postmortem examinations
Barnes [24]; Hinton and Kane [25]	*B. influenzae* (800)	3 doses of 0.5, 1, and 1.5 mL at 24-h intervals	Heat	3 locally isolated strains [24]
Duval and Harris [27]	*B. influenzae*[d]	3 doses at 3-d intervals	Chloroform	Old strain obtained from Rockefeller Institute (not locally and freshly isolated)
Wadsworth [28]	*B. influenzae* (1000)	Not reported	Not reported	15 strains obtained from Research Laboratories of New York City
Ely et al [29]	Hemolytic streptococci (250)	3 doses of 0.25, 0.5, and 1 mL at 48-h intervals	Heat	Multiple virulent strains obtained from patients in the camp

[a.] Some civilian population received a different vaccine from that used in military personnel. The first number and second number in the parenthesis were the amount of organisms for military personnel and some of the civilian populations, respectively.

[b.] The first number and second number in the parenthesis were for the first dose and the second dose, respectively.

[c.] Vaccines used earlier in the epidemic contained *B. influenzae.*

[d.] The dose for adults was one billion *B. influenzae* for the first injection, one-half this number for the second, and one billion for the third injections.

Table 5.2: Characteristics of the 12 included studies

Study	Country (population type)	Study period	Remarks
Cadham [15]	Canada (civilian and military)	October 1918 to February 1919	Military and civilian data were reported separately. Military data were from soldiers in Winnipeg, and influenza case patients were among hospitalized patients; civilian data were reported by 108 physicians in Manitoba and Saskatchewan.
Cherry [16]	Australia (civilian)	December 1918 to March 1919	Unlike other studies, this study did not examine the incidence of influenza among vaccinated and unvaccinated individuals; it analyzed data from 3891 patients with influenza treated in several hospitals in Melbourne, Australia. Patients from quarantine stations or with unknown vaccination status were excluded in the current analysis.
Erye and Lowe [18]	United Kingdom (military)	October–December 1918	Data from 15 units of New Zealand troops in the United Kingdom were included. Data from hospital B and camp G were included in the current analysis because the attack rate of pneumonia or case fatality of influenza was provided.
Leishman [19]	United Kingdom (military)	November 1918 to April 1919	Data from 24 military units in the United Kingdom were combined; numbers of influenza cases, complications, and deaths were derived from hospital records.
McCoy et al [20]	United States (civilian)	15 November to 9 December 1918	Alternate patients at a mental institution were vaccinated. Vaccination was completed 11 d before the occurrence of the first influenza case in this facility. The study population was aged <41 years.
Minaker and Irvine [21]	United States (civilian)	October–November 1918	Vaccinated individuals were military personnel or their civilian relatives and friends. Unvaccinated subjects were from the civilian population during the same period. Data from 4 locations were combined.
Rosenow and Sturdivant [22]	United States (civilian)	15 October 1918 to end of epidemic or 1 May 1919	Data were collected by distributing questionnaires to physicians supplied with the vaccine in Minnesota; reports from 530 physicians were fairly complete and were summarized. The observation period began on the day of the first inoculation.
Watters [23]	United States (civilian)	Not reported	Data from 5 commercial firms and 1 state hospital were combined.
Barnes [24]	United States (civilian)	22 October 1918 to end of epidemic	Data were from a state sanatorium at Wallum Lake, Massachusetts.
Duval and Harris [27]	United States (civilian)	15 October 1918 to January 1919	The majority of vaccinated subjects were employees in the large commercial houses, banks, and factories of New Orleans. Control subjects were those who refused to be vaccinated in these firms. Persons who had been sick before vaccination were excluded. Only individual groups A and B reported pneumonia data and were included in our analysis.
Hinton and Kane [25]	United States (civilian)	6 October to 30 November 1918	In an experiment at a state hospital for epileptics, patients in every other bed of a ward or room were vaccinated; vaccination was completed 6 d before the first influenza case occurred in this facility.
Wadsworth [28]	United States (civilian)	Not reported	Data included were from 146 laboratory staff of the New York State Department of Health.
Ely et al [29]	United States (military)	17 September to 21 October 1918	Data were combined from 7 military units at Puget Sound Navy Yard.

Table 5.3: The incidence of influenza among vaccinated and unvaccinated individuals with estimated risk ratio

Study	Population	Vaccinated	Unvaccinated	RR	P-value
Hilton [24]	Civilian	163/461 (35.4%)	178/ 518 (34.4%)	1.03 (0.87, 1.22)	0.79
McCoy [20]	Civilian	119/390 (30.5%)	103/390 (26.4%)	1.16 (0.92, 1.44)	0.23
Wadsworth [26]	Civilian	12/44 (27.3%)	27/102 (26.5%)	1.03 (0.58, 1.84)	1.00
Barnes [25]	Civilian	25/152 (16.4%)	23/113 (20.4%)	0.81 (0.48, 1.35)	0.42
Cadham [16]	- Military - Civilian	282/4842 (5.8%) 5203/52999 (9.8%)	238/2758 (8.6%) 21285/85941 (24.8%)	0.67 (0.57, 0.80) 0.40 (0.39, 0.41)	<0.0001 <0.0001
Minaker [21]	Civilian	111/6400 (1.7%)	43671/1233782 (3.5%)	0.49 (0.41, 0.59)	<0.0001
Erye [18]	Military	25/1817 (1.4%)	18/492 (3.7%)	0.38 (0.21, 0.68)	0.0021
Rosenow [22]	Civilian	13666/ 143760 (9.5%)	97258 / 345133 (28.2%)	0.34 (0.33, 0.34)	<0.0001
Leishman [19]	Military	221/15624 (1.4%)	2059/43520 (4.7%)	0.30 (0.26, 0.34)	<0.0001
Ely [27]	Military	144/4212 (3.4%)	1409/8486 (16.6%)	0.21 (0.17, 0.24)	<0.0001
Watters [15]	Civilian	89/1638 (5.4%)	471/1599 (29.5%)	0.18 (0.15, 0.23)	<0.0001
Duval [23]	Civilian	27/981 (2.8%)	130/338 (38.5%)	0.07 (0.05, 10.6)	<0.0001

Table 5.4: Attack rates of pneumonia and case-fatality rates among vaccinated and unvaccinated influenza patients and the corresponding risk ratios in studies using vaccines not containing pneumococci

Study	Population	Formula	Outcome	Vaccinated	Controls	RR	P-value
Duval [23]	Civilian	*B. influenzae*	Pneumonia	0/27	41/130	0.06	<0.0001
Hilton [24]	Civilian	*B. influenzae*	Death	28/163	24/178	1.27 (0.77, 2.11)	0.37
Barnes [25]	Civilian	*B. influenzae*	Death	4/25	9/57	1.01 (0.34, 3.98)	1.00
Wadsworth [26]	civilian	*B. influenzae*	Death	1/12	0/27	4.5 (0.16, 165)	1.00
Ely [27]	Military	Hemolytic streptococci	Death	0/144	96/1409	0.05 (0.003, 0.81)	<0.0001

Chapter 6 – Study #2

The anticipated incidence and severity of a "1918-like" influenza pandemic in contemporary populations: the contribution of antibacterial interventions

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Abstract

Background: The 1918 influenza pandemic was, and hopefully will remain, the most lethal infectious disease epidemic Humans have ever witnessed. While evidence suggests that the H1N1 virus responsible for the 1918 pandemic was particularly virulent, recent studies have shown that most of deaths were not caused by this virus alone but rather were due to secondary bacterial infections, primarily pneumococcal pneumonia. Given the availability of antibiotics and vaccines for pneumococcus as well as the flu virus, how would contemporary populations fare when we are next confronted with pandemic influenza due to a virus with the transmissibility and virulence of that of 1918?

Methods and Findings: To address this question we use a mathematical model and computer simulations. Our model considers the epidemiology of both the influenza virus and pneumonia-causing bacteria and allows for co-infection by these two agents as well as antibiotic treatment and prophylaxis. For our simulations we use influenza transmission and virulence parameters in the range estimated from 1918 pandemic data. We explore the anticipated rates of bacterial pneumonia and death in populations with different frequencies of pneumococcus carriage and contributions of antibiotic prophylaxis, treatment, and vaccination to these rates. Our analysis predicts that in countries with lower frequencies of pneumococcus carriage and access to antibiotics and vaccines, there would substantially fewer deaths due to pneumonia in contemporary populations confronted with a 1918-like virus than that observed in the 1918. Our results also predict that if the pneumococcus prevalence rates are less than 40%, these positive effects antibiotic prophylaxis and treatment would be manifest primarily at of level of individual. These interventions and the use of the

polyclonal conjugate pneumococcus vaccine would have little effect on the incidence of pneumonia in the population at large.

Conclusions: Pandemic preparedness plans should consider co-infection with and the prevalence of pneumococcus and other bacteria responsible for pneumonia. Although antibiotic prophylaxis and treatment can dramatically reduce the morbidity and mortality of pneumonia in individuals, save tor situations where pneumococcus is prevalent they will have little effect on the incidence of pneumonia in the population at large.

INTRODUCTION

Dominating our fears, driving our surveillance efforts and preparations for preventing, limiting the spread and treating influenza is the "Mother of all pandemics," the1918 flu [1]. Never in recorded history has the world confronted a single infectious disease pandemic that lead to as many deaths; estimates ranging from 20-100 million for the world at large, on the order of 675, 000 in the United States alone [2,3,4]. An estimated 28% of Americans were symptomatically infected by this virus [2] and, unlike most influenza pandemics, the rate of mortality was particularly high in people in their prime of life, those aged 18-40 years [1].

Can it happen again? Evidence from virus reconstruction and animal model experiments suggests that the H1N1 influenza virus responsible for the 1918 flu was more virulent than contemporary viruses of this hemagglutinin and neuraminidase serotype [3,4,5,6]. While we may not be able to say when, there is every reason to expect that the mutation and recombination events responsible for the evolution of influenza viruses with the combination of the virulence, and human to human transmissibility of the 1918 flu can and doubtless will be repeated.

Given what we know now about the 1918 influenza pandemic and the medical and public health technology currently available, in contemporary human populations what would be the incidence of symptomatic infections and mortality rate of a pandemic with an influenza virus of the virulence and transmissibility of that of 1918? What would be the optimum procedure to deal with this potential pandemic?

To address these questions, we use a mathematical model and computer simulations. Central to our model and analysis is the evidence that most of the pneumonias and deaths of the 1918 influenza can be attributed to a kind of conspiracy between the influenza virus and bacteria, primarily secondary infections with *Streptococcus pneumoniae* [7,8,9]. As evidence now indicates [10,11], in our co-infection model individuals infected both with the influenza virus and the bacteria have higher rates of mortality than those infected with the virus or bacteria alone. We calibrate our model by exploring the conditions required for it to account for dynamics and mortality rates observed in 1918, using virus transmission, pneumococcal carriage and virulence parameters estimated from the most realiable1918 data we can find. We then consider the incidence and mortality rates of secondary pneumococcal pneumonia that would be anticipated for a pandemic with a virus of the 1918 ilk with the pneumococcal carriage prevalence of contemporary populations in developed and developing countries with antibiotics for prophylaxis and treatment of secondary bacterial pneumonia. We discuss the implications of these computer simulation results to planning for the next influenza pandemic.

METHODS

Model development

Our complete "compartment" model [12] includes co-infection with the influenza virus and bacteria and antibiotic prophylaxis and treatment of the bacterial infection is obviously complex. To facilitate its presentation, we separately consider its different components and how they are modeled.

i) Single infection with the pandemic influenza virus: Considering a single homogenous population with no immunity to a novel pandemic strain, we assume that hosts are of four states with respect to the influenza infection, susceptible (X), asymptomatically infected (YF_A), symptomatically infected (YF_S) and recovered (ZF) (Figure 6.1A). The variables, X, YF_A, YF_S, ZF and those in the models to follow are both the densities of hosts of each of these states as well as their designations. The population size (N) is the sum of densities of all compartments. These and the other variables of this model and the models to follow as well as their parameters are separately defined in Table 6.1 and Table 6.2.

Both the YF_A and YF_S hosts are infectious, with transmission rate constants, β_{FA} and β_{FS} and a fraction, s_F ($0 \leq s_F \leq 1$) of newly infected hosts are symptomatic. Transmission occurs at rates proportional to product of X and λ_F, where λ_F is the sum of the products of the proportions of infected hosts and the corresponding transmission rate constants ($\lambda_F = \beta_{FA}YF_A/N + \beta_{FS}YF_S/N$). YF_A and YF_S hosts enter the recovered state (ZF) at rates ν_{FA} and ν_{FS} per host per day. In this, like most compartment models, virulence is reflected in the mortality rate. We assume symptomatically infected hosts (YF_S) have a death rate directly due to primary influenza infection d_F per host per day. The duration of the infections and thereby the amount of time available for transmission are the reciprocals of these rates, for example, symptomatic host, YF_S, remains infected for $1/(\nu_{FS} + d_F)$ days. The birth rate and influenza-independent death rate are neglected in our model.

ii) Single infection with bacteria: Given the variety of pneumococcal serotypes and

other bacterial pathogens, we assume that there is no immunity to bacterial colonization. As a result, our model for bacterial transmission only contains two compartments: susceptible (X) and colonized (YB) (Figure 6.1B). YB hosts are infectious with a transmission rate constant β_B and are spontaneously cleared at a rate of ν_B per host per day. In this model, we neglect the mortality due to the bacterial infection alone.

(iii)Virus – bacterial co-infection: For co-infection we separately consider hosts that are infected by both bacteria and virus and the order at which they are infected, bacteria first or virus first, YBF_A, YBF_S, YF_AB and YF_SB, respectively (Figure 6.1C). For example YBF_A represents hosts that are first colonized with bacteria and then asymptomatically infected with influenza virus. In this way we can allow for different rates of transmission and rates of recovery of the different jointly infected hosts. The purpose of making this distinction rather than considering only one class of joint infection is to account for the observations made with animal experiments. The likelihood of mortality is different in hosts first infected with the influenza virus than those first infected with the bacteria responsible for the pneumonia [10,11].

A YBF_A or YBF_S host can be produced by a YB host encountering one of the influenza infected hosts, YF_A, YF_S, YBF_A, YBF_S, YF_AB, and YF_SB. Similarly, a YF_AB or YF_SB host can be produced by a YF_A or a YF_S host being infected by a host carrying bacteria, YB, YBF_A, YBF_S, YF_AB, YF_SB, YP or ZFYB. We assume that influenza – infected hosts, YF_A and YF_S, are more likely to acquire bacterial colonization than influenza – free hosts when they encounter bacteria [13,14,15].

Therefore, a YF_A or YF_S host can be infected with bacteria at rate of $\delta_{FA} \times \lambda_B$ or $\delta_{FS} \times \lambda_B$, correspondingly, where δ_{FS} and δ_{FA} are constants ≥ 1 and λ_B is the sum of the products of the proportions of colonized hosts and the corresponding transmission rate constants (see Appendix for the equations). We also assume that co-infected hosts can transmit bacteria more efficiently than influenza – free hosts [16,17,18,19]. For example, co-infected hosts with symptomatic influenza (YBF_S and YF_SB) can transmit bacteria with a transmission rate constant $\sigma_{FS} \times \beta_B$ ($\sigma_{FS} \geq 1$). Similarly, YBF_A and YF_AB hosts have a transmission rate constant $\sigma_{FA} \times \beta_B$ ($\sigma_{FA} \geq 1$) for bacteria. On the other hand, we assume that the co-infected hosts have the same transmission rate constant for influenza virus, β_{FA} or β_{FS}, as YF_A or YF_S hosts, depending on whether their influenza infections are symptomatic or not.

The four different co-infected host populations YBF_A, YBF_S, YF_AB and YF_SB, leave their states at rates ν_{BFA}, ν_{BFS}, ν_{FAB}, and ν_{FSB} per host per day, respectively. Fractions of these co-infected hosts, respectively α_{BFA}, α_{BFS}, α_{FAB}, and α_{FSB} ($0 \leq \alpha s \leq 1$) develop secondary bacterial pneumonia (YP) and the remainder enter state designate ZFYB. In this state individuals have recovered from influenza, but are still colonized with bacteria because we are assuming the duration of infection and infectiousness for the influenza virus is much shorter than for the bacteria [20,21] . We also assume that jointly infected hosts, YBF_S and YF_SB have an additional death rate from primary influenza infection (d_F) as do the host symptomatically infected solely with the influenza virus, YF_S. Hosts with secondary bacterial pneumonia (YP) leave their compartment at rate ν_P per host per day. The case fatality of secondary bacterial pneumonia is c_P ($0 \leq c_P \leq 1$) and those who survive enter the ZFYB state. Hosts who recover from influenza infection (ZF and ZFYB) are assumed to have long-term

immunity to infection with this virus, do not return to the naïve uninfected host state X. On the other hand, we assume that immunity to influenza does not make these recovered ZF hosts any more refractory to bacterial colonization than X hosts.

vi) Co-infection model with antibiotic treatment and prophylaxis: Antibiotics would be used in two ways. One is to treat patients with secondary bacterial pneumonia. We assume that a fraction (f_T) of patients with secondary pneumonia, YP, will be treated with antibiotics. The treated people have a lower probability of death (case fatality), c_{PT} and their bacterial colonization is eliminated after treatment. The other way antibiotics would be used is for prophylaxis of hosts with symptomatic influenza to prevent secondary bacterial pneumonia. We assume that prophylaxis is empiric without distinction about whether the prophylaxed host has bacterial colonization or not. Thus, a fraction, f_P ($0 \le f_P \le 1$) of YF_S and YBF_S are prophylaxed with antibiotics. We assume that prophylaxed YF_S hosts have a lower probability of acquiring bacterial colonization once they encounter hosts carrying bacteria than unprophylaxed YF_S hosts. This efficacy of reducing susceptibility to colonization is represented by ρ ($0 \le \rho \le 1$). Therefore, YF_S hosts enter YF_SB at a rate $(1 - f_P)\delta_{FS}\lambda_B + f_P(1-\rho)\delta_{FS}\lambda_B$. For the prophylaxed YBF_S hosts, we assume that the efficacy of prophylaxis to clear the bacterial colonization is γ, and those who clear their bacterial colonization would move to the ZF state. In the remaining $(1 - \gamma)$, the prophylaxed hosts are still colonized with bacteria and we assume these individuals have the same risk of developing secondary pneumonia as unprophylaxed YBF_S hosts. Therefore, $(1 - \gamma)$ of the prophylaxed YBF_S hosts may develop secondary bacterial pneumonia with a probability of α_{FBS} or move to the ZFYB state. We assume that the prophylaxed hosts have the same additional death rate from primary influenza infection (d_F) as YF_S

hosts. In Figure 6.2, we illustrate how antibiotic prophylaxis is modeled for YBF_S hosts.

Parameterization:

Although our model is general and appropriate for most bacteria responsible for respiratory infections, for our numerical analysis of bacterial elements of the properties of this model we use parameters estimated for pneumococcus because pneumococci appears to be single most significant bacteria responsible for secondary infections in 1918, and the necessary epidemiological data seem to be most available for the pneumococci. The values or ranges of values of the parameters used in our models, as well as the sources of justification for these estimates are listed in Table 6.2.

The parameter d_F per host per day is the death rate (virulence) of the 1918 virus for symptomatic infected hosts in the absence of co-infection. The corresponding virulence parameters for co-infected hosts to develop secondary bacterial pneumonia are, α_{BFA}, α_{FAB}, α_{BFS}, and α_{FSB} for the YBF_A, YF_AB, YBF_S, and YF_SB host, respectively. We assume that asymptomatic influenza infections do not lead to bacterial pneumonia ($\alpha_{BFA} = \alpha_{FAB} = 0$). For symptomatic influenza infections, we allow for the possibility that influenza infection preceding pneumococcal colonization results in a higher risk bacterial pneumonia than bacterial colonization preceding influenza infection as the base case ($\alpha_{FSB} = 4\ \alpha_{BFS}$) [10,11]. To explore the sensitivity of the dynamics to this assumption, we also consider situations where $\alpha_{FSB} = \alpha_{BFS}$ and where $\alpha_{FSB} > \alpha_{BFS} = 0$. The values of the virulence - specific parameters for

the 1918 virus (d_F, α_{BFS}, and α_{FSB},) are calculated by determining the parameter conditions under which the co-infection model best accounts for the excess all-cause mortality in the New York City during the fall and winter wave of the1918 pandemic (5.3 per 1000) [22,23]. For this we assume that 7% of this excess mortality was caused directly by virus, with the remaining 93% due to bacterial pneumonia [7] and that pneumococcus was responsible for 71% of the bacterial pneumonias [9].

Given the major role played by the pneumococcus in pneumonia mortality during the 1918 pandemic, the likelihood of an infection with a virulent pneumococcus immediately after influenza becomes a critical risk for pneumonia. In 1918, it would seem that the likelihood of acquiring pneumococcus whilst suffering from influenza was greater than it is at present. The prevalence of pneumococcal carriage in adults in1918 was ~40% [24,25,26], whilst in contemporary populations in developed countries this carriage rate is less than 10% or even less than 5% [27,28,29,30]. It should be noted, however, that pneumococcal prevalence in adults is still very high in some developing countries, such as The Gambia where a 40% carriage has been reported [31]. Another difference between 1918 and today is the current widespread use of the pneumococcal conjugate vaccine (PCV) in children in developed countries, which has reduced the incidence of invasive pneumococcal disease and non-bacteremic pneumonia in all age group by approximately 45% [32,33]. To account for the PCV effect in our analysis, we reduce the values of α_{FSB} and α_{BFS} by 45% for contemporary populations [32,33]. The transmission rate constant of pneumococcus is not changed because its value depends on the equilibrium pneumococcal prevalence, which has not changed since the introduction of PCV, presumably because of the serotype replacement (Table 6.2) [34].

An overview of the analysis

After using our model to estimate values of the three virulence parameters of the 1918 influenza virus, we predict the incidence of pneumococcal pneumonia (IPP) under different scenarios about the prevalence of pneumococcal colonization at the start of a pandemic with a 1918-like influenza virus and different assumptions about the order of infection. We then investigate the extent to which antibiotic treatment for patients with secondary pneumonia can reduce the incidence and mortality of pneumococcal pneumonia. Finally, we consider the effect of antibiotic prophylaxis for patients with symptomatic influenza on reducing IPP and the pneumococcal prevalence. In this last analysis we explore the number of symptomatic influenza patients needed to be prophylaxed with antibiotics to prevent one case of pneumococcal pneumonia, Number Needed to be Prophylaxed (NNP).

$$NNP = \frac{1}{(AR_{Pneumonia|Flu,no\ propylaxis} - AR_{Pneumonia|Flu,100\%prophylaxis})}$$

Where $AR_{Pneumonia|Flu,no\ prophylaxis}$ and $AR_{Pneumonia|Flu,100\%\ prophylaxis}$ are the attack rates of secondary pneumococcal pneumonia among patients with symptomatic influenza patients given no prophylaxis and 100% prophylaxis, respectively.

We calculate NNP for different prevalences of pneumococcal colonization in populations with and without PCV programs. We also consider a range of values of the effective reproductive number of influenza (R_E) [21]), because the transmission of influenza virus could be mitigated by other interventions, such as antiviral prophylaxis or influenza vaccines. In our analysis, we are primarily interested in the

incidence of pneumococcal pneumonia rather than just the mortality rate. The reason is that the mortality rate reflects factors not considered in the model, like the quality of care or age of the patient. The incidence is also important as it reflects the number of people who need medication and hospitalization. To initiate these simulations, we assume that at the start of the pandemic, single YFs host are introduced into populations of 1,000,000 people who are wholly susceptible to influenza and different prevalences of pneumococcal carriage. We explore the sensitivity of the predicted NNPs by varying the central parameters by ± 10% and generating a tornado plot.

RESULTS:

Predicting and learning (estimating parameters) from the past:

We open our analysis of the properties of this model by exploring its ability to account for observations made in the 1918 pandemic, based on independent estimates of its parameters.

The 1918 influenza attack rate: At equilibrium, the fraction of population infected with influenza depends solely on the effective reproductive number R_E (roughly the number of secondary infections caused by a single infectious individual entering that population). When $R_E = 1.8$, the estimated value [58,59,60], in accord with our model 73% of the population would be infected with the virus. If we assume that 40% of these infected people (s_F) have typical influenza symptoms (see Table 6.2), the influenza attack rate would be 29%, which is close to that observed in the 1918 pandemic in the United States [2].

The virulence parameters: Assuming the excess mortality rate data for the 1918 pandemic in New York City, the above estimates of the influenza attack rate, and the other parameters in range of those in Table 6.2, using our co-infection model we determine the best fitting values of the three virulence parameters. We estimate the death rate due to the influenza virus alone, d_F, to be 0.00026 per day. The magnitudes of probabilities of developing secondary pneumonia by coinfected people, α_{FSB} and α_{BFS}, depend on the order of the infections. If we assume a prior symptomatic influenza infection increases the probability of pneumococcal pneumonia ($\alpha_{FSB} = 4\ \alpha_{BFS}$), α_{FSB} and α_{BFS} are respectively 14.4% and 3.6%. If the order of co-infection does not matter ($\alpha_{FSB} = \alpha_{BFS}$), the risk of secondary pneumonia for the co-infected hosts is 6.7%. In another extreme case, co-infected hosts who are first colonized with bacteria do not develop secondary pneumonia (α_{BFS}=0), the probability of developing secondary pneumonia for influenza first infection YF_SB hosts (α_{FSB}) is 23.0%.

Anticipating the Future

The effects of pneumococcus prevalence: Using baseline values of the parameters shown in Table 6.2, we estimate the incidence of pneumococcal pneumonia IPP for a future pandemic due to a 1918-like virus under different assumptions about the prevalence of pneumococcus colonization and the virulence of different orders of co-infection. The results of our analysis are presented in Figure 6.3A. If there is no order effect, $\alpha_{FSB} = \alpha_{BFS}$, IPP increases monotonically with the prevalence of pneumococcal prevalence. If there is an order effect, the IPP increases when the prevalence of pneumococcal carriage is low but declines when the prevalence of

carriage is high. The reason for this is that fewer people acquire new pneumococcal colonization during the pandemic. However, with respect to the IPP, these three assumptions yield very similar estimates when the prevalence of carriage is within the realistic range ($\leq$ 40%). Based on this prediction, we restrict the following analysis to a single situation ($\alpha_{FSB} = 4\ \alpha_{BFS}$). When the initial prevalences of carriage are 5%, 10%, 20% and 40% the predicted IPPs are, respectively 2.0, 3.8, 7.0 and 11.7. The mortality caused by primary viral infection does not vary with different pneumococcal prevalence and is approximately 0.37 per 1000 population.

Antibiotic treatment: We assume that antibiotic treatment reduces the case mortality rate of pneumococcal pneumonia from 30% to 10% (see Table 6.2). In Figure 6.3B we plot the anticipated incidence and mortality due to pneumococcal pneumonia for a 1918-like influenza pandemic as a function of the fraction of the treated patients with secondary pneumonia assuming 40% carriage. These results suggest that although wide-spread antibiotic treatment for pneumonia would significantly reduced mortality, it would have little effect on the IPP. The reason for this is that people with active pneumonia represent a small fraction of the individuals colonized with these bacteria and thereby responsible for their transmission. Thus, although treatment eliminates colonization as well as increases survival, its effect at the population level is anticipated to be small.

Antibiotic prophylaxis: In Figure 6.3C we consider the anticipated effects antibiotic prophylaxis on the IPP for different fractions of symptomatic influenza patients receiving these drugs prior to the onset of pneumonia. We make this

calculation for different initial prevalences of pneumococcus carriage. In this analysis we are assuming that the efficacy of antibiotic prophylaxis for reducing the susceptibility to bacterial colonization and clearance given colonization are respectively 78% and 72% [35]. As would be anticipated intuitively, antibiotic prophylaxis can substantially reduce the IPP. For example, with these parameters, 40% carriage and 75% of people with symptomatic influenza prophylaxed, the IPP would be reduced by more than 50%, relative to that anticipated in the absence of prophylaxis.

In Figure 6.3D, we follow the temporal changes in the prevalence of pneumococcus colonization during the course of the pandemic with different fractions of the population prophylaxed and an initial pneumococcal carriage prevalence of 40%. In the absence of antibiotic prophylaxis, pneumococcal prevalence gradually increases to 48.5% during the pandemic and then returns to the equilibrium level after the pandemic. Antibiotic prophylaxis would reduce bacterial transmission and thereby the level of pneumococcal carriage during the pandemic.

Pneumococcal Vaccination: To account for the wide spread use of the PCV in infants for our analysis, we assume the effect of the vaccine is to reduce the risk of secondary infection, lowering the 1918 estimates of α_{FSB} and α_{BFS} by 45%. To illustrate the consequences of this intervention, we consider the predicted IPP and the NNP (number needed to be prophylaxed) to prevent one case of pneumococcal pneumonia. We consider this for countries with and without PCV programs and for different effective reproductive numbe (R_E), see Table 6.3 and Table 6.4. When the R_E is 1.8,

the estimated NNP to prevent one case of pneumococcal pneumonia in countries without PCV program are 188.6, 98.8, 54.4 and 33.9 when the prevalence of bacterial carriage are respectively, 5%, 10%, 20% and 40%. The IPP is anticipated to be reduced by approximately 45% and the NNP increased by approximately 81% in countries with a PCV program relative to those without. The effective reproductive rate, R_E has marked effect on the estimated IPP, but the NNT is only slighted affected by the R_E. In coutries with a pneumococcal prevalence of 40%, no PCV program and no antiviral interventions to reduce R_E, the pandemic would not be very different from that of 1918 pandemic: the estimated IIP is 11.74 per 1000 and the NNP 33.9. On the other hand, in countries with only 5% pneumococcal prevalence and a PCV program, the estimated IPP is 1.08 per 1000 and NNP is 343 when the R_E is 1.8. If R_E is reduced to 1.2, e.g. by antiviral prophylaxis or vaccines, the esimtated IPP would be reduced to 0.40 per 1000 and the NNP 403.6.

Sensitivity analysis

To deal with the uncertainty of parameter values, we use a tornado plot to explore the sensitivity of our predicted NNP by varying the dominant parameters by ± 10% for a situation where the prevalence of bacterial colonization is 10% (Figure 6.4). The estimated NNP is most sensitive to the risks of secondary pneumonia among the co-infected people (α_{FSB} and α_{BFS}). Other influential parameters included the recovery rate for pneumococcal colonization (ν_B), the recovery rate for influenza (ν_{FS} and ν_{FA}), the effect of influenza infection on bacterial colonization and transmission (δ_{FS} and σ_{FS}), the efficacies of antibiotic prophylaxis on bacterial transmission and colonization (ρ and γ).

DISCUSSION:

"It's tough to make predictions, especially about the future."

(Attributed to Yogi Berra but also Neils Bohr)

Were the world confronted with a pandemic due to an influenza virus with a transmission rate, virulence and virulence mechanism similar to that of the 1918 H1N1 virus, would we better off now than we were then? We interpret the results of this theoretical study as support for a positive answer to this question.

Central to our model and this interpretation is the evidence that most of the morbidity and mortality of the 1918 pandemic can be attributed to secondary bacterial infections, primarily pneumonia due *Streptococcus pneumoniae* (pneumococcus). The evidence and arguments in support of this assertion have been presented elsewhere and won't be reviewed here [7,8,9,36,37,38,39,40]. Also central to our model and interpretation is the premise that influenza increases the likelihood of colonization by pneumococcus [13,14,15] and the rate of transmission of these bacteria [16,18,19,41]. Finally we assume that in the course of an influenza pandemic with the transmissibility and virulence of that of 1918, virtually all cases of pneumococcus pneumonia occur in co-infected people.

There are two primary reasons for anticipating substantially lower rates of the bacterial pneumonia responsible for most of the morbidity and mortality of the 1918 influenza pandemic, especially in current developed countries. First, in developed and many underdeveloped countries the prevalence of pneumococcus carriage is substantially lower than it was in 1918 [24,25,28,29,42,43,44]. As a result there

would be both lower rates of pneumonia and the infectious transmission of these bacteria. Second is the widespread use of the polyclonal conjugate vaccines PCV for pneumococcus. Although this vaccine appears to contribute little to the decline in the frequency of carriage of these bacteria due to serotype replacement [34], there is good evidence that PVC reduces the likelihood of pneumococcal pneumonia not only in vaccinated individuals, but as a consequence of heard immunity to others as well [32,33].

In many cases, interventions for infectious diseases that are good for individuals may have little positive and sometimes even may even negative consequences for the collective. The results of our analysis suggest this going to be the case for both antibiotic prophylaxis and treatment during a 1918-like influenza pandemic. Because of the relatively small risk of secondary bacterial infections in populations with low and modest prevalence of pneumococcus carriage, antibiotic prophylaxis for all symptomatic influenza patients would have little effect in reducing the incidence of pneumonia in the collective. In accord with our analysis, hundreds of patients with symptomatic influenza would need to be prophylaxed, NNP, to prevent a single case of secondary bacterial pneumonia. When considering this NNP and contribution of antibiotic use to the ascent of resistance, at the level of the collective antibiotic prophylaxis for all symptomatic influenza infections would be difficult to justify. This is particularly so when antibiotic treatment for the bacterial pneumonias that do arise in this small minority of is a viable alternative to prophylaxis for many.

In this regard, a very different conclusion may be in order for underdeveloped

countries where the prevalence of pneumococcus carriage is substantial [31]. Because of the latter, the estimated NNP to prevent a single case of secondary pneumonia would be on the order of 30 - 35. Unfortunately, associated with high frequencies of pneumococcal carriage in these countries is a dearth of the money needed for the wide spread purchase prophylactic antibiotics. No matter where, the cost effectiveness of antibiotic prophylaxis would greatly augmented if there were procedures to identify people who at particular risk of these secondary infections or members of clear risk groups, like people with other co-morbidities.

Antibiotic treatment of secondary bacterial infections would also be more advantageous to individuals than populations. In accord with our analysis, the treatment of patients with pneumococcus pneumonia would have a negligible affect on the transmission and thereby the frequency of carriage and infection by these bacteria. Unlike prophylaxis, however, the individual benefit of the use of antibiotics for treatment can be considerable and will almost certainly outweigh the cost associated with the promotion of resistance

If, as suggested by the animal model experiments [10,11], the likelihood of pneumonia in humans is greater when the bacteria follow the virus infection than the reverse, the order of the infection would play an important role in the course of the disease for individuals. Our results suggest, however that this order effect may contribute little to the incidence of bacterial pneumonia for the population at large. As long as the prevalence of carriage is modest, less than 40%, the incidence of pneumococcal pneumonia, IPP, is relatively independent of the order of infection (see Figure 6.3A). On the other hand, when the prevalence of pneumococcus carriage is greater than 40%, the order of infection becomes increasingly important at the

population as well as the individual level. In fact, as the prevalence increases bacterial colonization can be protective if the likelihood of pneumonia is greater when the viral infection precedes the bacterial. That is, as the frequency of carriage increase, a greater fraction of people infected with the influenza virus would already be colonized with pneumococcus.

As complex as our model might seem, it captures little of the real complexity of the epidemiology of influenza, bacterial pneumonia and the prevention and treatment of these diseases in human populations. Contrary to what we assumed in our model: (i) Human populations are not homogeneous and have multiple subpopulations. The rates of transmission, prevalence of pneumococcus carriage and the parameters governing course of the infection and co-infection are not going to be the same for all subpopulation. Age, life-style, social contact pattern, local density and physical condition will all contribute to the values of these parameters. Also contributing to this variation is immune state of these hosts due to prior encounters with influenza viruses and pneumococci that are antigenically the same or cross reacting with those encountered during the pandemic. (ii) Pneumococci are not homogenous. There is great deal of genetic variation in *S. pneumoniae* including variation in the capsule structure, their serotype, of which are 93 at last count [45]. This underlying variation will certainly contribute to individual differences in the infection parameters as will the extent of coverage by the poly- but much less than 93- valent vaccines.

While we can incorporate these other complexities into our model, at this stage we don't see much justification in doing so. There are two reasons for this, one

practical and one philosophical. Estimates of the parameters of this extended model are not available. Although we could generate numerical solutions to the large numbers of equations in a more complex and realistic model, without the constraints of parameter values in a realistic range it would be difficult to interpret the implications of the results of this analysis. This interpretation problem would be further confounded by the vast numbers interactions between different elements of this model.

The philosophical justification for not expanding the complexity of these models is their role in this endeavor. In an essay about model building in population biology written more than a half century ago [46], Richard Levins argued that there are three properties of a mathematical model we want to maximize, reality, generality and precision. He postulated that we only able to maximize two at a time. To address this general question about the morbidity and mortality of a pandemic with a 1918-like influenza virus in contemporary populations, reality and generality are more important than precision. Moreover, because of the relative dearth of estimates of parameters and the problems of interpreting complex models, reality and generality are the best we can achieve at this time.

While our model is general for any combination of directly transmitted viruses and bacteria, we restricted our numerical analysis of its properties to only a single species of bacteria, *Streptococcus pneumoniae.* These are not the sole bacteria known to be responsible for bacterial pneumonia during the 1918 influenza pandemic or anticipated to be so in future pandemics. Part of our justification for focusing on

pneumococcus in this is by default. Estimates of the necessary parameters are more available for pneumococcus than other bacteria responsible for pneumonia. Another justification is the relative prevalence of the different species of bacteria responsible for these pneumonias. A review of antemortem cultures from normally sterile sites of pneumonia patients in the 1918 pandemic showed that respectively *Streptococcus pneumoniae*, *Streptococcus pyogenes* (Group A Streptococcus) and all other bacteria comprised 71%, 28% and 1% of positive cultures [9].

In contemporary populations pneumococcus remains the predominant bacteria responsible for community-acquired bacterial pneumonia [47]; group A Streptococcus is rare as a source of these pneumonias (0-1%), athough it was commonly associated with measles and influenza outbreaks in the pre-antibiotic era [48,49,50]. Postmortem culture studies suggest that *Staphylococcus aureus* pneumonia is a significant source of mortality following influenza in contemporary populations. We suggest that to some extent this observation is the product of sampling bias. Because *S. aureus* pneumonias are more likely to be mortal than those due to pneumococcus [51,52] and because of concern about the incidence of antibiotic resistance in Staphylococcus, these bacteria may be more likely to be cultured in postmortems of antibiotic-treated patients. Most importantly, *S. aureus* pneumonias are primarily nosocomial and less likely to be responsible than pneumococcus for the community-acquired pneumonias that are the focus of our models. Be all this as it may, as noted our model is a general analogue of epidemiology of viral – bacterial co-infection. By changing the parameter values, it can be applied to any combination of directly transmitted viruses and bacteria.

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Appendix: Differential equations for the complete model with co-infection, antibiotic treatment and prophylaxis.

$$\frac{dX}{dt} = -(\lambda_B + \lambda_F) \times X + \nu_B \times YB$$

$$\frac{dYB}{dt} = \lambda_B \times X - \nu_B \times YB - \lambda_F \times YB$$

$$\frac{dYF_A}{dt} = (1 - s_F) \times \lambda_F \times X - \nu_{FA} \times YF_A - \lambda_B \times \delta_{FA} \times YF_A$$

$$\frac{dYF_S}{dt} = s_F \times \lambda_F \times X - (\nu_{FS} + d_F) \times YF_S - \lambda_B \times \delta_{FS} \times (1 - f_P) \times YF_S - \lambda_B \times \delta_{FS} \times (1 - \rho) \times f_P \times YF_S$$

$$\frac{dYF_AB}{dt} = \lambda_B \times \delta_{FA} \times YF_A - \nu_{FAB} \times YF_AB$$

$$\frac{dYF_SB}{dt} = \lambda_B \times \delta_{FS} \times (1 - f_P) \times YF_S + \lambda_B \times \delta_{FS} \times (1 - \rho) \times f_P \times YF_S - (d_F + \nu_{FSB}) \times YF_SB$$

$$\frac{dYBF_A}{dt} = (1 - s_F) \times \lambda_F \times YB - \nu_{BFA} \times YBF_A$$

$$\frac{dYBF_S}{dt} = s_F \times \lambda_F \times YB - (d_F + \nu_{BFS}) \times YBF_S$$

$$\frac{dYP}{dt} = \alpha_{FAB} \times \nu_{FAB} \times YF_AB + \alpha_{FSB} \times \nu_{FSB} \times YF_SB + \alpha_{BFA} \times \nu_{BFA} \times YBF_A + \alpha_{BFS} \times \nu_{BFS} \times (1 - f_P) \times YBF_S$$
$$+(1 - \gamma) \times \alpha_{BFS} \times \nu_{BFS} \times f_P \times YBF_S - \nu_P \times YP$$

$$\frac{dZF}{dt} = \nu_{FA} \times YF_A + \nu_{FS} \times YF_S - \lambda_B \times ZF + \nu_B \times ZFYB + f_T \times (1 - c_{PT}) \times \nu_P \times YP$$
$$+\gamma \times \nu_{BFS} \times f_P \times YBF_S$$

$$\frac{dZFYB}{dt} = (1 - \alpha_{FAB}) \times \nu_{FAB} \times YF_AB + (1 - \alpha_{FSB}) \times \nu_{FSB} \times YF_SB + (1 - \alpha_{BFA}) \times \nu_{BFA} \times YBF_A$$
$$+(1 - \alpha_{BFS}) \times \nu_{BFS} \times (1 - f_P) \times YBF_S + (1 - \alpha_{BFS}) \times (1 - \gamma) \times \nu_{BFS} \times f_P \times YBF_S$$
$$+(1 - f_T) \times (1 - c_P) \times \nu_P \times YP + \lambda_B \times ZF - \nu_B \times ZFYB$$

where

$$\lambda_F = (\beta_{FA} \times YF_A + \beta_{FS} \times YF_S + \beta_{FA} \times YF_AB + \beta_{FS} \times YF_SB + \beta_{FA} \times YBF_A + \beta_{FS} \times YBF_S) \div N$$

$$\lambda_B = \beta_B \times (YB + \sigma_{FA} \times YF_AB + \sigma_{FS} \times YF_SB + \sigma_{FA} \times YBF_A + \sigma_{FS} \times YBF_S + YP + ZFYB) \div N$$

$$N = X + YB + YF_A + YF_B + YF_AB + YF_SB + YBF_A + YBF + YP + ZF + ZFYB$$

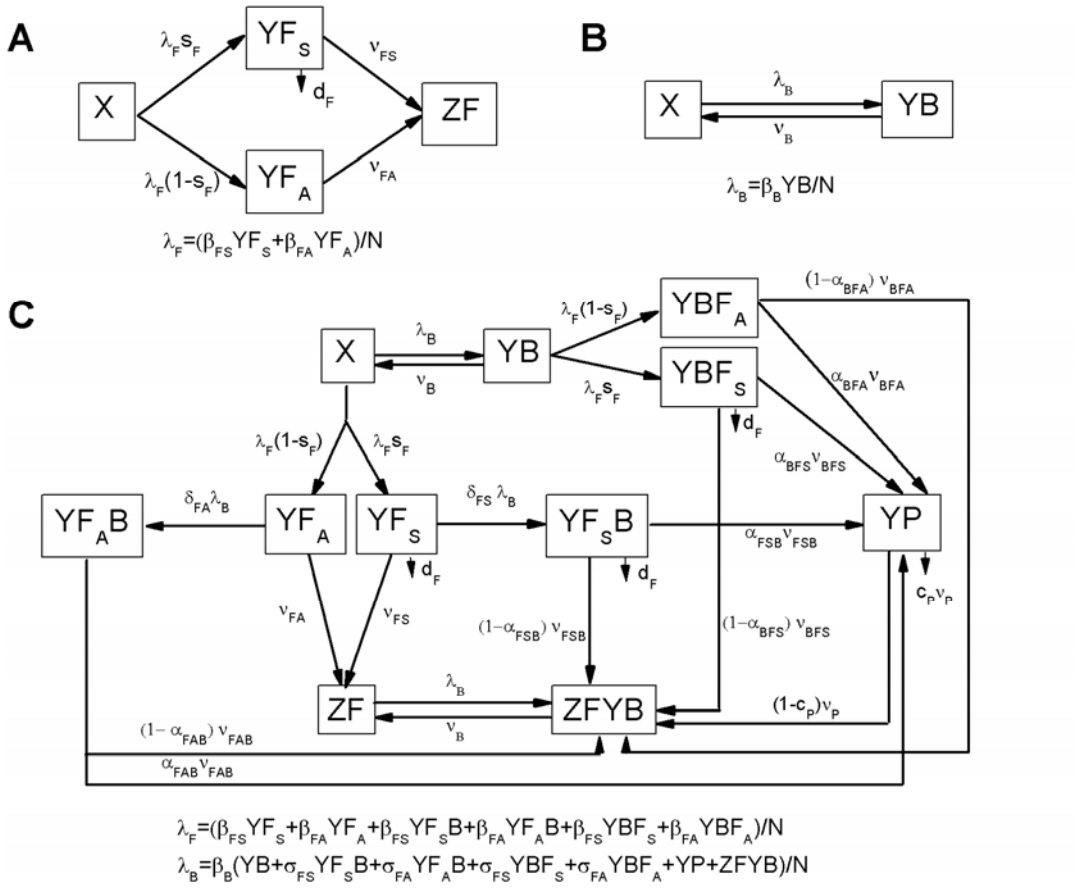

Figure 6.1. Model structure

(A) Compartment model for single infection with pandemic influenza virus. (B) Compartment model for single infection with bacteria. (C) Compartment model for virus – bacterial co-infection in influenza pandemics. See Table 6.1 and Table 6.2 for definition of the variables and parameters, and see the text for more details about the model description.

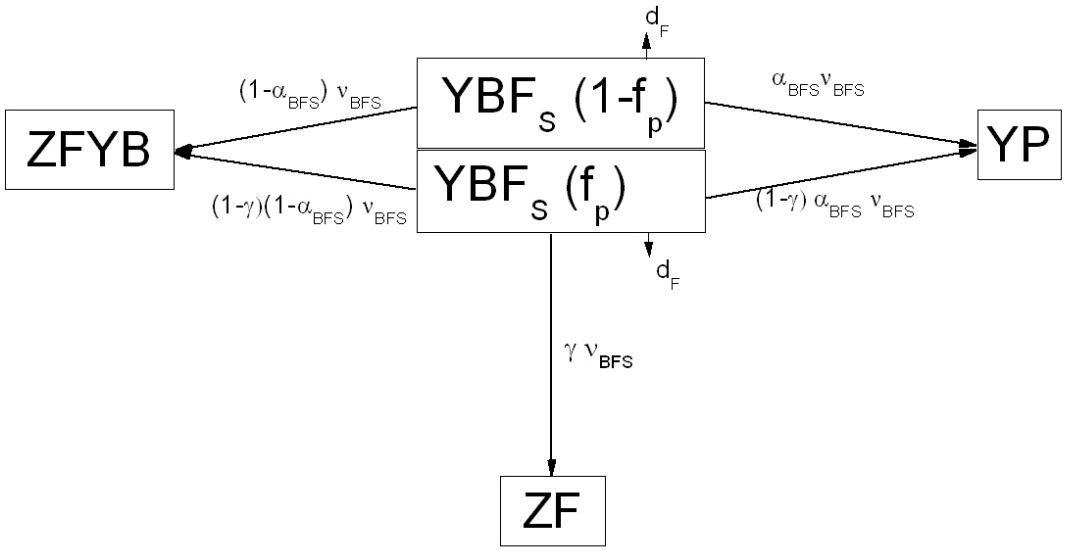

Figure 6.2. Diagram for how antibiotic prophylaxis is modeled for YBF_S hosts. See the text for more details.

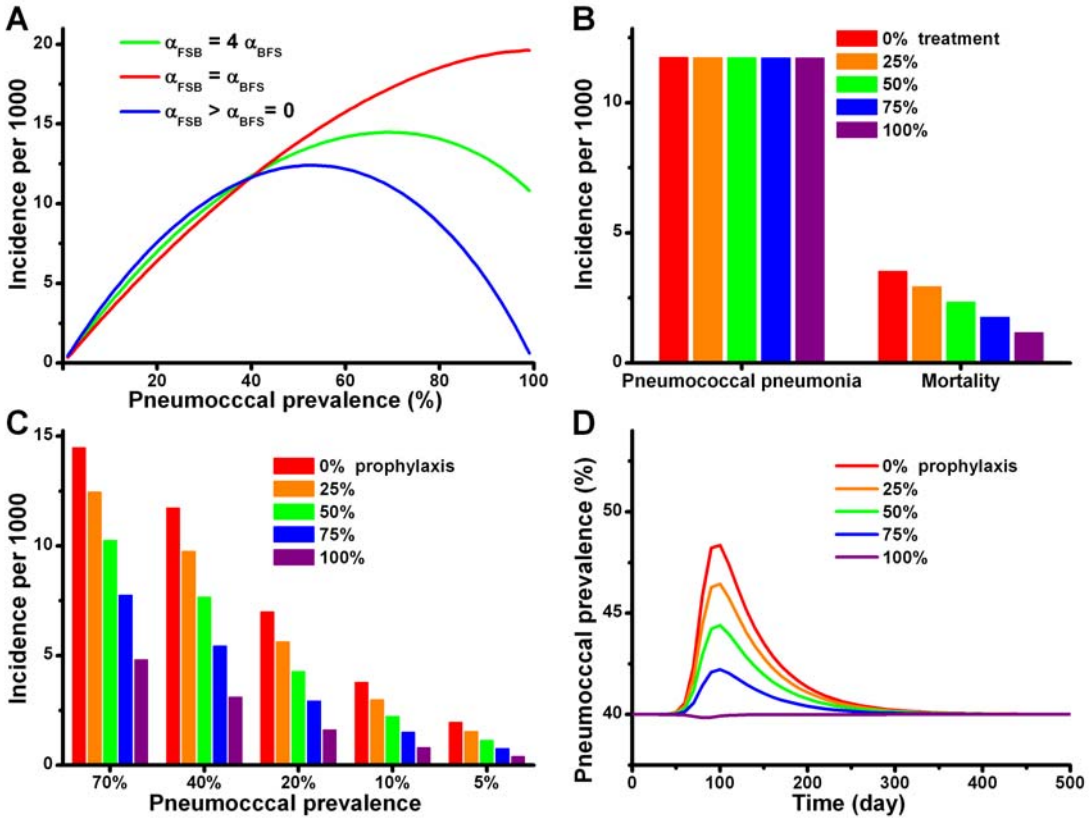

Figure 6.3. Modeling results.

(A) The predicted incidence of pneumococcal pneumonia in a 1918-like influenza pandemic under different initial prevalence of pneumococcal colonization and three assumptions regarding the relationship between α_{FSB} and α_{BFS}. (B) The predicted mortality and incidence of pneumococcal pneumonia in a 1918-like pandemic when 0%, 25%, 50%, 75% and 100% of pneumonia patients were treated with antibiotics and the initial pneumococcal carriage was 40%. (C) The predicted incidence of pneumococcal pneumonia in a 1918-like pandemic when 0%, 25%, 50%, 75% and 100% of patients with symptomatic influenza infection received antibiotic prophylaxis under different initial pneumococcal prevalence. (D) The predicted prevalence of pneumococcal colonization during the progress of a 1918-like influenza pandemic when 0%, 25%, 50%, 75% and 100% of patients with symptomatic influenza infection received antibiotic prophylaxis.

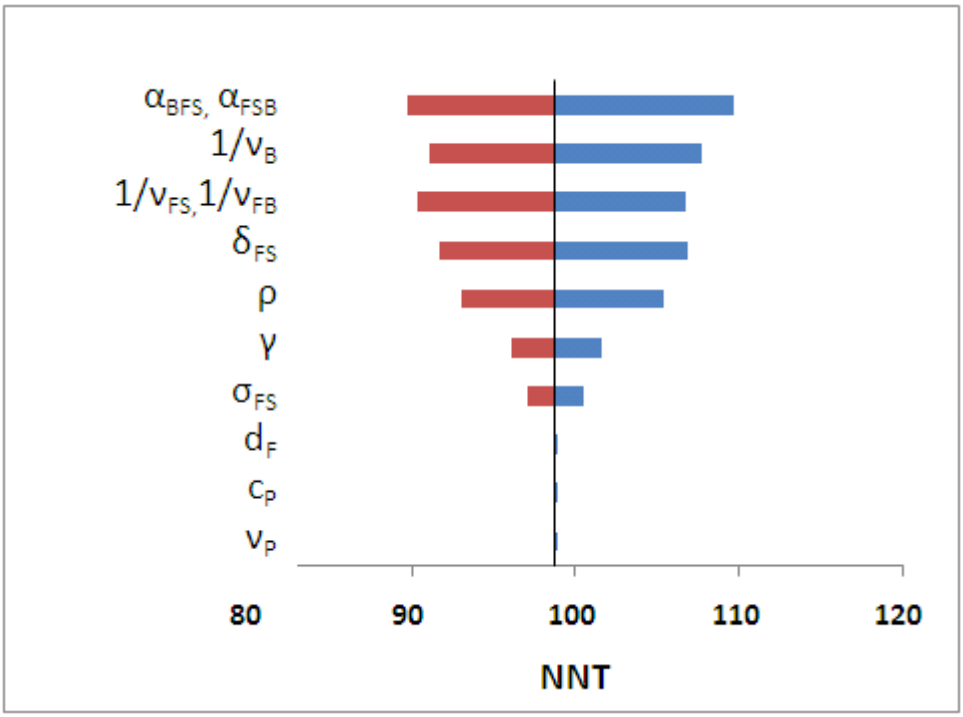

Figure 6.4. Sensitivity analysis. Tornado plot of number needed to be prophylaxed (NNP) to prevent one case of pneumococcal pneumonia with ± 10% changes in parameters when the initial pneumococcal prevalence is 10%.

Table 6.1: Variables in the influenza virus – bacterial co-infection model

Variables	
X	Number of people susceptible to both influenza virus and bacteria
YF_A	Number of people with asymptomatic influenza infection but not colonized with bacteria
YF_S	Number of people with symptomatic influenza infections but not colonized with bacteria
ZF	Number of people have recovered from influenza infection
YB	Number of people colonized with bacteria and susceptible to influenza virus
YBF_A	Number of co-infected people who are colonized with bacteria first then acquire asymptomatic influenza infection
YBF_S	Number of co-infected people who are colonized with bacteria first then acquire symptomatic influenza infection
YF_AB	Number of co-infected people who are asymptomatically infected with influenza first and then acquire bacterial colonization
YF_SB	Number of co-infected people who are symptomatically infected with influenza first and then acquire bacterial colonization
YP	Number of people who develop secondary bacterial pneumonia
ZFYB	Number of people who have recovered from influenza infection but are still colonized with bacteria.
N	Total number of population

Table 6.2: Parameters in the influenza – bacteria co-infection model

Symbol	Meaning	Base case	Assumptions / References
R_E	Effective reproductive number for pandemic influenza virus	1.8	Based on Refs. [53,54,55]. Can be reduced with antiviral interventions
s_P	Proportion of newly influenza-infected hosts who have typical influenza symptoms	40%	Although 66.9% of influenza infection results in some symptoms [20], we decided to use 40% to get an influenza attack rate similar to those observed in 1918 [2]. Additionally, this number is close to fraction of infected people with typical influenza symptoms (like fever) [20] who are more likely to be prophylaxed.
ν_{FS}, ν_{FB}	Recovery rates per host per day for YF_A and YF_B hosts	1/4.8	Based on Ref. [20]. Assume $\nu_{FS} = \nu_{FB}$
β_{FA} β_{FS}	Transmission rate constant for hosts with asymptomatic and symptomatic influenza infection.	4.96 7.92	Calculated from R_E , $\nu_{FS,}$ and s_P. Assume asymptomatic hosts are half infectious as symptomatic hosts ($\beta_{FA} = 0.5*\beta_{FS}$).
d_F	Death rate per host per day directly due to influenza virus among hosts with symptomatic influenza infection	**0.00026**	**Virulence parameter estimated by calibration**
p_B	Prevalence of bacterial colonization before the pandemic	40%	The prevalence of pneumococcal colonization was 40% in 1918 [24,25,26]. Varied for different scenarios today
β_B,	Transmission rate constant for bacteria	$\nu_B/(1- p_B)$	Assume bacterial transmission before the pandemic is at equilibrium, thus $\beta_B = \nu_B/(1- p_B)$. Varied based on p_B.
ν_B	Recovery rate per host per day for bacterial colonization	1/37	Based on Ref. [21]
δ_{FA}	The increase of bacterial acquisition for hosts with asymptomatic influenza infection	1	Assume asymptomatic influenza infection does not increase the susceptibility to bacterial colonization.
δ_{FS}	The increase of bacterial acquisition for hosts with symptomatic influenza infection	4	Based on an animal study showing that influenza infection increased the susceptibility of ferrets to pneumococcal acquisition [56].
σ_{FA}	The increase of transmission of bacteria for hosts with asymptomatic influenza infection	1	Assume asymptomatic influenza infection does not increase bacterial transmission
σ_{FS}	The increase of transmission of bacteria for hosts with symptomatic influenza infection	3.5	Based on a human study testing the dispersal Staphylococcus aureus after experimentally infected with rhinovirus [18]
ν_{BFS}, ν_{BFA}, ν_{FSB}, ν_{FAB}	Recovery rates per host per day for YBF_S, YBF_A, YF_SB and YF_AB, respectively	1/4.8	Assume equal to ν_{FS} and ν_{FB} because the duration of influenza infection is much shorter than the duration of bacterial colonization.
$\alpha_{BFA}, \alpha_{FAB}$	Risk of secondary bacterial for YBF_A and YF_AB	0	Assume people with asymptomatic influenza infections do not develop secondary bacterial pneumonia.
α_{BFS} α_{FSB}	Risk of secondary bacterial for YBF_S and YF_SB.	**3.6%** **14.4%**	**Virulence parameters estimated by calibration**. Assume $\alpha_{FSB} = 4\ \alpha_{BFS}$ in the base case but also consider two extreme conditions: (i)$\alpha_{FSB} = \alpha_{BFS}$; (ii)$\alpha_{FSB} > \alpha_{BFS} = 0$. These numbers are reduced by 45% in countries with PCV program for children.
ν_P	Recovery rate per host per day for secondary bacterial pneumonia	1/10	Based on Ref. [9]
c_P	Case fatality rate of secondary pneumococcal pneumonia	30%	Based on Ref. [2]
f_T	Fraction of symptomatic flu patients treated with antibiotics	0 – 100%	Varied for different scenarios
c_{PT}	Case fatality rate of secondary pneumococcal pneumonia for patients treated with antibiotics	10%	Based on Ref. [57]
f_P	Fraction of symptomatic flu patients prophylaxed with antibiotics	0 – 100%	Varied for different scenarios
ρ	The efficacy of antibiotic prophylaxis in reducing bacterial acquisition	78%	Based on based on a clinical trial testing the effect of short-course, high-dose oral amoxicillin therapy on pneumococcal carriage [35] .
γ	The efficacy of antibiotic prophylaxis in clearing pneumococcal colonization	72%	

Table 6.3: The estimated incidence of pneumococcal pneumonia (IPP) per 1000 in countries with and without a PCV program under different pneumococcal prevalence and R_E

The estimated Incidence of pneumococcal pneumonia (IPP) per 1000						
	R_E= 1.8		R_E= 1.5		R_E= 1.2	
Pneumococcal carriage	No PCV	PCV	No PCV	PCV	No PCV	PCV
5%	1.96	1.08	1.47	0.81	0.73	0.40
10%	3.78	2.08	2.85	1.57	1.42	0.78
20%	7.00	3.85	5.31	2.92	2.68	1.47
40%	11.74	6.45	9.05	4.98	4.68	2.57
70%	14.48	7.96	11.45	6.29	6.13	3.37

Table 6.4: The number needed to treat to prevent one case of pneumococcal pneumonia (NNT) in countries with and without a PCV program under different pneumococcal prevalence and R_E

The estimated number needed to treat to prevent one case of pneumococcal pneumonia (NNT)						
	R_E= 1.8		R_E= 1.5		R_E= 1.2	
Pneumococcal carriage	No PCV	PCV	No PCV	PCV	No PCV	PCV
5%	188.6	343.0	201.6	366.5	222.0	403.6
10%	98.8	179.6	105.1	191.1	115.1	209.3
20%	54.4	99.0	57.4	104.4	62.1	112.9
40%	33.9	61.7	35.2	63.9	36.9	67.1
70%	30.4	55.2	30.5	55.5	30.6	55.6

Chapter 7 – Study #3

The nasopharyngeal interaction of *Streptococcus pneumoniae, Haemophilus influenzae* and *Staphylococcus aureus* among young children living in the Peruvian Andes: comparison of detection using culture and Real-time quantitative polymerase chain reaction

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Support:

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55-character title: Nasopharyngeal bacterial colonization in Peru and qPCR

44-character title: Nasopharyngeal bacterial colonization in Peru

ABSTRACT

Background: *Streptococcus pneumoniae, Haemophilus influenzae* and *Staphylococcus aureus* are commonly carried in children's nasopharynx and may influence the colonization of each other. They are usually detected by culture; however, real-time quantitative polymerase chain reaction (qPCR) may complement data from traditional culture techniques.

Methods: We compared results of culture and qPCR for detection of these three bacteria in 485 nasopharyngeal samples collected from 382 healthy young children from a prospective cohort study in the Peruvian Andes. Patterns of concurrent colonization among these bacteria were studied using repeated measures logistic regression models with generalized estimating equations. Spearman correlation coefficients were used to assess correlations between bacterial densities.

Results: In detection of colonizing bacteria, qPCR had a higher yield than culture. As the bacterial density measured by qPCR increased, the sensitivity of culture increased. We found a positive association between *S. pneumoniae* and *H. influenzae* colonization using qPCR (OR=1.87 – 1.97, $p<0.01$). The densities of *S. pneumoniae* and *H. influenzae* were positively correlated (Spearman correlation coefficient 0.32, $p<0.001$). A negative association was found between *S. pneumoniae* and *S. aureus* among only among children without symptoms of acute respiratory infections when using qPCR (OR=0.6, $p<0.05$).

Conclusion: Use of qPCR improved the yield on nasopharyngeal samples from young children studies based on culture and can provide quantification data. The

observed colonization patterns suggest that vaccines and antimicrobials that target specific nasopharyngeal bacteria may unexpectedly influence the rest of the bacterial flora.

INTRODUCTION

Several common respiratory bacterial pathogens, such as *Streptococcus pneumoniae, Haemophilus influenzae* and *Staphylococcus aureus* reside in the human nasopharynx without causing symptoms. However, they can occasionally invade adjacent sites or the blood stream and cause respiratory or systemic disease such as otitis media, pneumonia, bacteremia and meningitis. The pneumococcus is the leading bacterial pathogen associated with these diseases,[1] whereas *H. influenzae* is a common cause of acute otitis media[2] and *S. aureus* is an emerging cause of clinically important infections (especially methicillin – resistant *S. aureus*)[3], ranging from mild skin infections and sinusitis to severe diseases such as pneumonia, bacteremia, and endocarditis.

The reported colonization prevalence of these bacteria varies widely, which may be due to both the differences in study populations with respect to age and socioeconomic status and variations in sampling and detection methods. Previous studies using conventional culture have shown that 13 – 85% and 6 – 80% of children carry *S. pneumoniae* and *H. influenzae* in their nasopharynx, respectively.[2, 4] The prevalence of *S. aureus* colonization in the nose or nasopharynx has been found to be 10 – 35% in children.[5-7] However, we are unaware of data on the prevalence of these three bacteria among young children in the Peruvian Andes.

Understanding factors that influence nasopharyngeal colonization by these bacteria is essential since colonization is the initial step for the development of disease. In addition, nasopharyngeal colonization of these pathogens in children is an important source of horizontal transmission to other individuals in the community.[8] Whether

bacteria colonize or not is determined by a complex combination of factors including host characteristics that influence the exposure or susceptibility to specific bacterial species and direct interactions between different bacteria. Host factors that have been suggested to influence the colonization prevalence of *S. pneumoniae, H. influenzae,* or *S. aureus* include age, gender, ethnicity, immunity, crowding, number of siblings, daycare attendance, season, antibiotic therapy, acute respiratory infections, and environmental exposure to tobacco smoke.[2, 4, 9-11]

Furthermore, different bacterial species may interact with each other by competing for resources and by producing chemicals or by inducing host immune responses that influence the growth of other bacteria in the nasopharynx.[12] Several studies using culture have shown that *S. aureus* prevalence is negatively associated with *S. pneumonia* prevalence,[13, 14] especially with vaccine-type (VT) pneumococci,[4, 7, 15] while some other studies found no association between them.[16, 17] Colonization by *S. pneumoniae* and *H. influenzae* has been positively associated.[10, 13, 17]

Differences in detection methods may greatly influence reported nasopharyngeal colonization prevalence.[18] Culture, the most commonly used method to detect and quantify bacteria, has several drawbacks, such as low sensitivity compared with molecular methods, and it is time-consuming and laborious to perform quantification analysis. Real-time quantitative polymerase chain reaction (qPCR) is potentially a more sensitive and rapid alternative to culture.[18] However, direct comparisons of the performance of culture and qPCR for detection bacteria in nasopharyngeal samples among healthy children are limited.

The goals of our study were to (1) compare culture and qPCR for detection of *S. pneumoniae, H. influenzae, or S. aureus* in nasopharyngeal samples; (2) describe the prevalence of nasopharyngeal colonization by *S. pneumoniae, H. influenzae,* and *S. aureus* in young children in rural communities of the Peruvian Andes; and (3) investigate the interaction between *S. pneumoniae, H. influenzae,* or *S. aureus* in two ways: evaluate whether colonization status (presence/absence) of one bacterium influences the colonization status of the other two bacteria, and evaluate whether the densities of the three bacteria are correlated.

MATERIALS AND METHODS

Study population and data collection

The population for this study was derived from a prospective cohort study of ~500 children 0-35 months of age in the District of San Marcos, Cajamarca, Peru. The study aimed to investigate whether indoor air pollution and acute respiratory infections (ARI) influenced nasopharyngeal colonization with *S. pneumoniae* in healthy children. Children aged <3 years were enrolled and information on demographic, socio-economic, and the presence of symptoms ARIs/pneumonia symptoms was collected at baseline and at weekly visits to the homes by trained field workers. A new episode of ARI was defined as the presence of cough and/or fever. Routine nasopharyngeal swabs for bacterial colonization were collected monthly on all children. Nasopharyngeal samples were collected with using Rayon swabs and the swab was immediately placed in 1 ml of transport medium (skim-milk tryptone glucose glycerol, STGG) processed at a local laboratory according to WHO standards and stored at -70°C. Enrollment into this study was done between May and August 2009. This study included 485 consecutive nasopharyngeal samples collected between

August and September 2009..

Bacteriologic cultures

To increase the sensitivity of cultures, 200 µl of STGG sample were enriched in THY broth (Todd-Hewitt broth supplemented with 0.5% of yeast extract) containing 1% of rabbit serum and incubated for 6 h at 37°C with 5% CO_2.[18] To identify *S. pneumoniae* strains, the enrichment broth was inoculated onto blood agar plates (BHI agar containing 5% sheep blood) and incubated overnight at 37°C in a 5% CO_2 atmosphere.[18] Presumptive pneumococcus-like colonies were confirmed by the optochin susceptibility test and bile solubility. Equivocal results were confirmed by PCR using primers that target the *cps*A gene[19] and DNA extracted from the *S. pneumoniae* isolates using the Chelex method (see below).[20]

For detection of *S. aureus*, the enriched THY broth was inoculated onto mannitol salt agar and incubated at 37°C in 5% CO_2 for 24 hours; colonies morphologically suggestive of *S. aureus* were confirmed by performing PCR on Chelex-extracted DNA using published primers targeting the *nuc* gene.[21]

For detection of all *H. influenzae*, 200 µl of STGG sample was enriched in brain heart infusion broth with 5% Fildes enrichment (BD Diagnostics, NJ, USA) for 6 h at 37°C in 5% CO_2; the enriched broth was then plated onto chocolate agar with bacitracin and incubated overnight. The presence of *H. influenzae* was confirmed by performing PCR using the primers targeting the *hpd* gene which detect all *H. influenzae* stains[22] on DNA extracted from suspected colonies using the extraction method previously described by LaClaire *et al.*[23]

DNA extraction from cultured isolates using the Chelex method for PCR and multiplex PCR

A loopful of bacteria from the culture plate was placed into a 1.5 ml micro-centrifuge tube and mixed with 200 µl of 5% Chelex-100 resin (Bio-Rad) and 2 µl of Proteinase K (20mg/ml, QIAgen).[20] After incubation at 56ºC for an hour then at 95ºC for 10 minutes, the sample was mixed and then centrifuged at 13,000 rpm for 5 minutes to completely separate the layers. The DNA-containing supernatant was used as template in PCR reactions.

Multiplex PCR for molecular serotyping for *S. pneumoniae*

To define the serotypes or serogroups of *S. pneumoniae* isolates, a multiplex PCR approach that detects all pneumococcal capsular serotypes was used.[19] This approach included eight sequential reactions; each reaction contains 5 pair of primers and an internal control that targets the *cps*A locus, present in all *S. pneumoniae* isolates.[19] DNA extracted from multiple *S. pneumoniae* serotypes were included as controls in each multiplex PCR reaction. The multiplex PCRs were performed in 25 µl volumes, with each reaction mixture containing the following: 2.5 µl of DNA-containing bacterial lysate, 1X PCR master mix (Qiagen Multiplex PCR kit) and the specific set of primers as reported.[19, 24] Thermal cycling was performed in the MyCycler system (Bio-Rad) under the following conditions: 94°C for 15 min followed by 35 amplification cycles of 94°C for 1 minute, 54°C for 1.5 minute, and 72°C for 1 minute. PCR products were run in 2% agarose gels and visualized under UV light after ethidium bromide staining. Serotypes 6A, 6B, and 6C were differentiated by PCR method described by Jin *et al*.[25] Since these PCR-based serotyping could not distinguish 9V/9L and 18A/B/C/F, these serotypes were considered vaccine-type

pneumococci.

DNA extraction for qPCR from the original nasopharyngeal specimen and reference strains

Two-hundred µl of STGG sample were added with 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer containing 0.04 g/ml lysozyme and 75 U/ml of mutanolysin and then incubated for 1 h at 37°C in a water bath. Using the QIAamp DNA Mini protocol, DNA was eluted in 100 µl of elution buffer and kept at -70°C. Genomic DNA from the reference strains of *S. pneumoniae* (ATCC 33400 or TIGR4),[26] *S. aureus* (ATCC 25923) and *H. influenzae* type b (CDC reference strain M5216) was also extracted from overnight cultures using the QIAamp kit. DNA concentrations were measured by Nanodrop method (Nanodrop Technologies, Wilmington, DE) and serial dilutions in DNase-, RNase-free water were made to obtain the qPCR standards.

Real-time quantitative PCR (qPCR)

The total density of *S. pneumoniae*, measured in CFU/ml, was determined using pre-optimized concentrations of the forward primer (5'-ACGCAATCTAGCAGATGAAGCA-3'; 200 nM), reverse primer (5'-TCGTGCG TTTTAATTCCAGCT-3'; 200 nM), and probe (5'-FAM-TGCCGAAAACGCTTGATACAG GGAG-3-BHQ1; 200 nM) targeting the *lytA* gene as describe previously.[26] To create standard curves, purified genomic DNA of *S. pneumoniae* reference strain, in the range of 10 fg to 10 ng, was used, assuming a genome size of 2.1 Mb; therefore, the qPCR standards represented 4, $4x10^1$, $4x10^2$, $4x10^3$, $4x10^4$, $4x10^5$ or $4x10^6$ CFU.

The total density of *S. aureus*, measured in CFU/ml, was determined using the forward primer (5'-GTTGCTTAGTGTTAACTTTAGTTGTA-3'; 800 nM), reverse primer (5'- AATGTCGCAGGTTCTTTATGTAATTT-3'; 800 nM), and probe (5-FAM- AAGTCTAAGTAGCTCAGCAAATGCA-3-BHQ1; 400 nM) targeting on *nuc* gene.[21] To create standard curves, purified genomic DNA of *S. aureus* reference strain, in the range of 10 fg to 10 ng, was used, assuming a genome size of 2.8 Mb; therefore, the qPCR standards represented 3, $3x10^1$, $3x10^2$, $3x10^3$, $3x10^4$, $3x10^5$ or $3x10^6$ CFU.

The total density of *H. influenzae*, measured in CFU/ml, was determined using recently published primers and probe: forward primer (5'-GGTTAAATATGCCGATGGTGTTG-3'; 100 nM), reverse primer (5'-TGCATCTTTACGCACGGTGTA-3'; 300 nM), and probe (5'-HEX-TTGTGTACACTCCGT "T-BHQ1" GGTAAAAGAACTTGCAC-3'; 100 nM) targeting the *hpd* gene.[22] To create standard curves, purified genomic DNA of *H. influenzae* reference strain, in the range of 10 fg to 10 ng was used, assuming a genome size of 1.8 Mb; therefore, the qPCR standards represented 5, $5x10^1$, $5x10^2$, $5x10^3$, $5x10^4$, $5x10^5$ or $5x10^6$ CFU.

Real-time quantitative PCR was performed using the Bio-Rad CFX96^{TM} Real-Time PCR Detection System (Hercules, CA, USA) in a reaction volume of 25 µl containing the EXPRESS qPCR Supermix Universal (Invitrogen by Life Technology, CA, USA), 2.5 µl of sample DNA, forward and reverse primers and fluorogenic probe with concentrations described above. For *S. pneumoniae*, the qPCR conditions were 95°C

for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min; samples with cycle threshold (Ct) values ≤ 35 were considered positives. For *H. influenzae*, the cycling conditions included 50°C for 2 min, 95°C for 10 min, and 45 cycles of 15 s at 95°C followed by 1 minute at 60°C; and samples with Ct values ≤ 35 were considered positives. For *S. aureus*, the conditions were 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Positive samples were samples with Ct values ≤ 38.

Statistical analysis:

All statistical analyses were performed using SAS version 9.1 (SAS institute, Inc, Cary, NC, USA). To assess whether colonization by one bacterial species was associated with colonization by the other two bacterial species, repeated measures logistic regression models with generalized estimating equations (GEE) were used because some children contributed more than one swab to the analyses. We modeled colonization by *S. pneumoniae, H. influenzae* and *S. aureus* separately, and each model included variables presenting the presence/absence of the other two bacterial species as the main exposures of interest. Covariates to be controlled for potential confounding included age in months, gender, the presence of acute respiratory infection within two weeks of sample collection and antibiotic usage within the past 7 days of sample collection. Two-way interaction terms of exposure variables as well as exposure variables and covariates were added to the model using backward elimination and a cutpoint of p-value < 0.1; if the model did not run with all interaction terms in, stepwise selection was used instead. The analyses using bacterial culture and using qPCR to determine the colonization status of each bacterium were performed separately.

To assess the degree of correlation between densities of each pair of bacteria, we used Spearman correlation coefficients. This test was preferred because bacterial densities determined by qPCR were not normally distributed and contained a large fraction of zero (qPCR negative). We then focused on samples which were positive for two bacteria and re-calculated Spearman correlation coefficients. Positive samples for each bacterium were also categorized into high density and low density using median as a cutoff. Chi-square tests were performed to examine whether there was an association among bacterial density classified as high or low of the three bacteria.

Ethical Approvals

This study was approved by the Ethical Review Boards (ERB) of the Instituto de Investigación Nutricional, Vanderbilt University and Emory University. An ERB approved written informed consent form was obtained from one parent (usually the mother) of participating subjects at enrollment. The study was also approved by the local health authorities, and by community leaders.

RESULTS

Study population

A total of 485 consecutive nasopharyngeal samples from 382 children aged 0 – 36 months (mean 17.7, median 16.7 months) were included in this analysis. Fifty-three percent of the children were male. Among the enrolled children, 329 children had vaccination information available, and the percentages of children who received at least one dose of PCV and Hib immunization before the swabs were collected were 8.8% and 66.3%, respectively. Of the 485 swabs, 39 (8.0%) were collected within two weeks of the presence of ARI symptoms, and 13 (2.6%) were collected from children

taking antibiotics within the past 7 days of sample collection, respectively.

Comparison of culture and qPCR

For *H. influenzae*, 485 samples were tested using qPCR while only 381 samples were cultured because of inadequate amount of STGG samples left. Comparison of bacterial culture and qPCR showed indicated that 61.0% and 77.5% of the swabs were positive for *S. pneumoniae*, 24.1% and 38.8% for *H. influenzae* and 10.9% and 40.6% for *S. aureus* (Table 7.1). For all three bacteria, culture-positive swabs were also positive by qPCR and qPCR-negative swabs were also negative by culture. As the bacterial density measured by qPCR increased, the isolate rate of culture also increased (Table 7.2). The proportion of samples indicating colonization by single or multiple species, by culture and qPCR, are shown in Table 7.3. Results of cultures showed that none, 1, or 2 bacteria species were present in 27.3%, 50.4% or 22.3% of NP swabs respectively, while qPCR detected none, 1, or 2 bacteria species in 7.9%, 39.5% and 52.6% of NP swabs, respectively.

Prevalence and incidence of colonization by *S. pneumoniae, H. influenzae,* and *S. aureus*

Among the first swabs collected from 382 children, the prevalence of colonization by *S. pneumoniae*, *H. influenzae,* and *S. aureus* was 77.2%, 37.7%, and 39.5.0% determined by qPCR. For 103 children who contributed two swabs taken one month apart, 47.4% of 19 children who were originally not colonized by *S. pneumoniae* acquired pneumococcal colonization after one month, and 14.3% of 84 children who were originally colonized by *S. pneumoniae* cleared the colonization as determined by qPCR. The percentages of acquisition and clearance after one month were 29.0% and

34.1% for *H. influenzae*, and 43.8% and 54.5% for *S. aureus*, respectively.

Assessing the associations between the colonization status of *S. pneumoniae, H. influenzae,* and *S. aureus*

Repeated measures logistic regressions models predicting colonization (presence or absence) by *S. pneumoniae*, *H. influenzae*, and *S. aureus* determined by cultures are shown in Table 7.4. The model predicting colonization by *S. pneumoniae* indicated that colonization by *H. influenzae* was positively associated with *S. pneumoniae* (OR 2.69, 95% CI 1.55 – 4.69, $p < 0.001$), while the presence of *S. aureus* was negatively associated with colonization by *S. pneumoniae* (OR 0.45, 95% CI 0.23 – 0.90, $p = 0.023$), controlling for age, gender, acute respiratory infections, and antibiotics usage. A positive association between *S. pneumoniae* and *H. influenzae* was also indicated in the model predicting the colonization by *H. influenzae* (OR 2.70, 95% CI 1.55 – 4.69, $p < 0.001$). Probably because of low prevalence of *S. aureus* detected by cultures, the model with *S. aureus* colonization as the dependent variable did not run when other potential confounders were included in the model; the model not controlling for confounders did not indicate any association between bacteria. Multiplex PCR revealed that 38.2% of the S. pneumoniae isolates were vaccine serotypes (VT) covered in heptavalent pneumococcal conjugate vaccine. We did not find a significant association between *vaccine-type S. pneumoniae* and *S. aureus* or *H. influenzae* colonization, probably because of lower power if only VT pneumococci were considered (data not shown). A significant positive association between non-VT *S. pneumoniae* and *H. influenzae* was indicated in the model predicting the colonization by *H. influenzae* (OR 1.69, p=0.042).

Table 7.5 shows the results from repeated measures logistic regressions models predicting colonization by *S. pneumoniae*, *H. influenzae*, and *S. aureus* determined by qPCR. The model with *S. pneumoniae* as the dependent variable contained a significant interaction term of *S. aureus* (p=0.028) and ARI, which indicated a positive association between *S. pneumoniae* and *H. influenzae* (OR 1.97, 95% CI 1.22 – 3.18, p = 0.005) and a significant negative association with *S. aureus* only among children without ARI (OR 0.60, 95% CI 0.39 – 0.94, p = 0.025). The model predicting *H. influenzae* colonization also showed a positive association between *H. influenzae* and *S. pneumoniae* (OR 1.87, 95% CI 1.15 – 3.04, p = 0.012). The model predicting the colonization by *S. aureus* also showed that *S. pneumoniae* was negatively associated with *S. aureus* only among children without ARI (OR 0.62, 95% CI 0.39 – 0.99, p = 0.046). These models might seem to suggest that that ARI modified the association between *S. pneumoniae* and *S. aureus*. However, , the number of observations in some cells of the 2 x 2 table for children with ARI was small (Table 7.6); therefore, our was too small to adequately examine the association of *S. pneumoniae* and *S. aureus* in the presence of ARI.

Assessing the correlation of bacterial densities of *S. pneumoniae, H. influenzae* and *S. aureus*

The Spearman correlation coefficients for the correlation of densities between *S. pneumoniae* and *H. influenzae*, between *S. pneumoniae* and *S. aureus*, and between *H. influenzae* and *S. aureus* were 0.32 (p <0.001), -0.07 (p=0.122), and -0.08 (p = 0.089), respectively. If only positive swabs (density > 0) were considered, the Spearman correlation coefficients were 0.39 (p<0.001), -0.01 (p=0.876) and -0.10 (p=0.413), respectively.

When the density of the positive swabs was categorized into high and low for each bacterium, swabs with high density of *S. pneumoniae* were more likely to have high density of *H. influenzae* (OR 3.84, p<0.001). No association was found between S. *pneumoniae* and *S. aureus* and between *H. influenzae* and *S. aureus* categorized into high and low densities.

DISCUSSION

In this study, we describe the colonization of young children in the rural Andes using molecular methods to complement information from traditional cultures. Our data indicate that molecular approaches greatly increase the yield of detection of bacterial colonization, suggesting that these molecular assays may become the assays of choice to detect bacterial colonization in the nasopharynx. A limitation of this approach is that no non – culture based serotyping method has been validated to date so we used a combination of culture and molecular serotyping to perform the pneumococcal serotyping. When comparing the results of culture and qPCR for detection of *S. pneumoniae, H. influenzae* and *S. aureus* for nasopharyngeal samples stored in the WHO standard medium for detection of pneumococci in the nasopharynx (STGG),[27] we found that the sensitivity of cultures increased as the bacterial density increased. Culture had lower sensitivity than qPCR for detection of these bacteria, especially when bacterial density was low, and its performance may be affected by factors associated with the growth of different isolates or the presence of other competing bacterial species. The much higher detection rate using qPCR than using culture suggests that previous studies using culture alone may have underestimated the prevalence of bacterial colonization in the nasopharynx. However, qPCR could detect both viable and nonviable bacterial cells, which may also lead to an overestimation of

bacterial colonization prevalence. It is still to be determined whether or when we need to use qPCR to detect more nasopharyngeal colonization of bacteria because those qPCR-positive but culture-negative samples usually have low bacterial density or contain non-viable cells. However, qPCR provides a rapid quantification; one important possible implication is that qPCR can help to distinguish infection and colonization because bacterial density may be higher during infection than during colonization.[28-31] Several recent studies have used molecular techniques with high sensitivity to detect pneumococcal colonization because accurate detection of pneumococcal colonization is important for evaluation and formulation of pneumococcal vaccines.[18] To our knowledge, this is the first study to use qPCR to examine the prevalence of *H. influenzae* and *S. aureus* colonization in healthy children. However, the clinical and epidemiologic relevance of using qPCR for detection of *H. influenzae* and *S. aureus* in nasopharyngeal samples is still to be determined.

The negative association between *S. pneumoniae* and *S. aureus* colonization observed in this study is consistent with previous reports in Europe, Asia, America and Africa.[4, 7, 13-15] This association has also been observed in children with ARI and pneumonia,[32, 33] but our study could not examine whether the association changed with the presence of ARI or not because only a small proportion of swabs were collected during ARI in our study. One possible mechanistic explanation for this bacterial interference phenomenon is the bactericidal effect of hydrogen peroxide produced by *S. pneumoniae*[34] and thus colonization by *S. pneumoniae* may "protect" the host against *S. aureus* colonization. The negative association between *S. aureus* and *S. pneumoniae* was not found in children in HIV-infected children,[13, 33] suggesting that host

immunologic factors can influence the interaction of bacteria in the nasopharynx. It is possible that upper respiratory viral infection may also lead to an immunological disruption of the regulation of carriage of *S. aureus* versus the pneumococcus. Future studies which include more children with ARI are required to examine whether ARI modifies the association between *S. aureus* and *S. pneumoniae*.

Our study lacked sufficient power to separately consider VT or non-VT *S. pneumoniae* because we only were able to serotype the cultured isolates and the prevalence of *S. aureus* colonization determined by culture was low in our study population. Some studies have suggested that this negative association is only evident for VT pneumococci,[4, 7, 15] which may account for the observed increased in *S. aureus* related acute otitis media in a PCV randomized trial[35] and the observed increase of bacteremia caused by *S. aureus* after the introduction of PCV.[36] However, one other study has also show that non-VT *S. pneumoniae* is negatively associated with *S. aureus*.[13] If this is true, PCV programs will have smaller effects on *S. aureus* colonization because the overall prevalence of pneumococcal colonization has not changed since the widespread use of PCV due to serotype replacement.[37, 38] It is possible that some of the non-VT strains have stronger inhibitory effects against *S. aureus* than other non-VT strains, and thus the effect of PCV on *S. aureus* colonization may depend on the distribution of non-VT *S. pneumoniae* after the widespread use of PCV.

Similar to several other epidemiologic studies,[10, 13, 17] we found a positive association between colonization of *S. pneumoniae* and *H. influenzae*, and in addition we find that

their densities in the nasopharynx were positively correlated. This is consistent with a recent study showing that the presence of *H. influenzae* increases pneumococcal biofilm formation in vivo and in vitro.[39] However, in vitro experiments show that hydrogen peroxide and neuraminidase produced by *S. pneumoniae* can inhibit the growth of *H. influenzae*.[40, 41] Studies in mice suggest that the immune response primarily elicited by *H. influenzae* reduces the density of some pneumococcal strains,[12, 42] while the presence of *S. pneumoniae* facilitate the colonization by a new *H. influenzae* population.[12] Another epidemiologic study showed that these two bacteria were negatively associated in children with ARI, but the association shifted from negative to positive in the presence of *M. catarrhalis*.[32] Therefore, the mechanism by which *S. pneumoniae* and *H. influenzae* influence each other in the nasopharynx is complex and may be affected by which one initially colonized the nasopharynx, the host immune response, the presence of ARI symptoms, and other bacterial species present in the nasopharynx.

The observed association between bacteria in our study as well as in other epidemiologic studies may be a result of direct bacterial interaction or due to unmeasured host-specific confounders that simultaneously influence colonization status of different bacteria. Jacoby *et al.* recently used a hierarchical multivariate logistic model to analyze longitudinal data and simultaneously model colonization statues of different pathogens as dependent variables, trying to differentiate the host-level interaction and microbe-level interaction.[17] Interestingly, they found a positive association between *S. pneumoniae* and *H. influenzae* at host-level only among aboriginal children, not among non-aboriginal children, and they found neither host-level nor microbe-level association between *S. pneumoniae* and *S. aureus*, which

is contrary to many previous studies. We could not use their approach to analyze our data because only a small subset of children contributed more than one swab. Although we have controlled many host-level potential confounders, we cannot be sure that the observed associations were truly bacterial interactions.

Our study has several other limitations. Other pathogens potentially involved in interactions with the three bacteria we examined, such as *M. catarrhalis* [32] and coagulase-negative staphylococci,[11] were not studied. In addition, information on other potential confounders, such as number of siblings, family size and daycare attendance was only available on 64% of study children. However, the association between the three bacterial species did not seem to be confounded by these factors in analyses confined to the subset of children from whom complete information was available.

Understanding how bacteria interact with each other in the nasopharynx, no matter whether it is synergistically or competitively, is essential for designing preventive measures. This is especially true in this era of vaccines and antimicrobials which target specific bacteria and may unexpectedly influence the bacterial flora. Our study confirmed the previous observed negative association between *S. pneumoniae* and *S. aureus* and positive association between *S. pneumoniae* and *H. influenza*e using culture and qPCR to test nasopharyngeal samples collected from young children in rural communities of the Peruvian Andes. Our study demonstrates for the first time the impact of bacterial density on the detection of these bacteria by culture and the relationship between the density of pneumococcal and haemophilus colonization. As colonization density may be an essential precursor to disease caused by these bacteria

our study suggests that future colonization studies of these pathogens should also measure bacterial density.

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Table 7.1: Comparison of bacterial culture and qPCR for detection of *Streptococcus pneumoniae, Staphylococcus aureus,* and *Haemophilus influenzae* in nasopharyngeal swabs.

	No. (%)								
	S. pneumoniae			***H. influenzae***			***S. aureus***		
	Culture (-)	Culture (+)	Total	Culture (-)	Culture (+)	Total	Culture (-)	Culture (+)	Total
qPCR(-)	109 (22.5%)	0 (0.0%)	109 (22.5%)	233 (61.2%)	0 (0.0%)	233 (61.2%)	288 (59.4%)	0 (0.0%)	288 (59.4%)
qPCR(+)	80 (16.5%)	296 (61.0%)	376 (77.5%)	56 (14.7%)	92 (24.1%)	148 (38.8%)	144 (29.7%)	53 (10.9%)	197 (40.6%)
Total	189 (39.0%)	296 (61.0%)	485 (100.0%)	289 (75.9%)	92 (24.1%)	381 (100.0%)	432 (89.1%)	53 (10.9%)	499 (100.0%)

Table 7.2: The percentage of culture positive swabs, stratified by bacterial density determined using qPCR.

	No. of Culture positive / No. in density categories (%)		
Bacterial density (CFU/ml)	***S. pneumoniae***	***H. influenzae***	***S. aureus***
0	0/109 (0.0%)	0/233 (0.0%)	0/288 (0.0%)
$0 – 10^4$	75/144(52.1%)	0/2 (0.0%)	1/45 (2.2%)
$10^4 – 10^5$	140/151 (92.7%)	1/22 (4.6%)	12/102 (11.8%)
$10^5 – 10^6$	75/75 (100%)	13/36 (36.1%)	18/25 (72.0%)
$> 10^6$	6/6 (100%)	78/88 (88.6%)	22/25 (88.0%)
Total	296/485 (61.0%)	92/381 (24.1%)	53/485 (10.9%)

Table 7.3: Distribution of *Streptococcus pneumoniae, Staphylococcus aureus,* and *Haemophilus influenzae* in 499 nasopharyngeal swabs

	Culture results (n=381)	qPCR results (n=381)
none	104 (27.3%)	31 (7.9%)
S. pneumoniae	151 (39.6%)	104 (26.7%)
S. aureus	22 (5.8%)	35 (9.0%)
H. influenzae	19 (5.0%)	15 (3.8%)
One species total	192 (50.4%)	154 (39.5%)
S. pneumoniae + *S. aureus*	17 (4.5%)	113 (29.0%)
S. pneumoniae + *H. influenzae*	67 (17.6%)	81 (20.8%)
S. aureus + *H. influenzae*	1 (0.3%)	11 (2.8%)
Two species total	85 (22.3%)	205 (52.6%)
S. pneumoniae + *S. aureus* + *H. influenzae*	0 (0%)	0 (0%)

Table 7.4: Estimated OR and 95% CI from repeated measures logistic regression models predicting the presence of *Streptococcus pneumoniae, Staphylococcus aureus,* and *Haemophilus influenzae* by culture

	OR (95% CI)[1]		
Predictors	*S. pneumoniae*	*H. influenzae*	*S. aureus* [2]
S. pneumoniae			
Absent (ref)	—	1	1
Present		**2.70 (1.55 – 4.69)**	0.57 (0.17 – 1.89)
H. influenzae			
Absent (ref)	1	—	1
Present	**2.69 (1.55 – 4.69)**		0.63 (0.20 – 2.00)
S. aureus			
Absent (ref)	1	1	—
Present	**0.45 (0.23 – 0.90)**	0.69 (0.29 – 1.66)	
Age (1-mo increase)	1.00 (0.98 – 1.02)	1.02 (0.99 – 1.05)	—
Sex			
Female (ref)	1	1	—
Male	1.36 (0.88 – 2.12)	0.65 (0.40 – 1.07)	
ARI			
Absent (ref)	1	1	—
Present	1.65 (0.68 – 3.99)	1.29 (0.54 – 3.10)	
Antibiotics			
No (ref)	1	1	—
Yes	1.34 (0.30 – 6.04)	1.74 (0.40 – 7.50)	

[1]OR, odds ratio; CI, confidence interval. Significant ORs and 95% CI are shown in boldface. Model for colonization of each bacterium included variables representing presence/absence of the other two bacteria and six potential confounders: age, sex, the presence of acute respiratory infection (ARI), antibiotic usage within the past 7 days (Antibiotics).
[2] The model predicting the colonization of *S. aureus* did not allow controlling for potential confounders because low prevalence of this species detected by culture.

Table 7.5: Estimated OR and 95% CI from repeated measures logistic regression models predicting the presence of *Streptococcus pneumoniae, Staphylococcus aureus,* and *Haemophilus influenzae* by real-time quantitative polymerase chain reaction (qPCR)

	OR (95% CI)[1]				
Predictors	*S. pneumoniae*[2]		*H. influenzae*	*S. aurues*[3]	
S. pneumoniae				ARI(-)	ARI (+)
Absent (ref)	—		1	1	1
Present			**1.87 (1.15 – 3.04)**	**0.62 (0.39 – 0.99)**	5.23 (0.57 – 48.0)
H. influenzae					
Absent (ref)	**1**		—	**1**	
Present	**1.97 (1.22 – 3.18)**			0.77 (0.53 – 1.14)	
S. aureus	ARI(-)	ARI(+)			
Absent (ref)	1	1	1	—	
Present	**0.60 (0.39 – 0.94)**	4.70 (0.79 – 27.9)	0.71 (0.49 – 1.04)		
Age (1-mo increase)	1.00 (0.98 – 1.02)		1.01 (0.99 – 1.03)	0.99 (0.97 – 1.01)	
Sex					
Female (ref)	1		1	1	
Male	1.11 (0.70 – 1.76)		0.75 (0.50 – 1.11)	0.73 (0.50 - 1.06)	
ARI	pneumococci (-)	pneumococci (+)		pneumococci (-)	pneumococci (+)
Absent (ref)	1	1	1	1	1
Present	0.53 (0.20 – 1.41)	4.13 (0.89 – 19.1)	1.74 (0.90 – 3.35)	0.15 (0.02 – 1.32)	1.30 (0.60 – 2.79)
Antibiotics					
No (ref)	1		1	1	
Yes	1.76 (0.25 – 12.4)		3.04 (0.76 – 12.1)	1.65 (0.52 – 5.26)	

[1]OR, odds ratio; CI, confidence interval. Significant ORs and 95% CIs are shown in boldface. Model for colonization of each bacterium included variables representing presence/absence of the other two bacteria and six potential confounders: age, sex, the presence of acute respiratory infection (ARI), antibiotic usage within the past 7 days (Antibiotics).

[2] The model predicting the colonization of *S. pneumoniae* included a significant interaction term of *S. aureus* and ARI.

[3] The model predicting the colonization of *S. aureus* included a significant interaction term of *S. pneumoniae* and ARI.

Table 7.6: The association between *Streptococcus pneumoniae* and *Staphylococcus* during stratified by the presence/absence of acute respiratory infections

	ARI (+)			ARI (-)		
	S. aureus (+)	*S. aureus* (-)	Total	*S. aureus* (+)	*S. aureus* (-)	Total
S. pneumoniae (+)	14	17	31	131	214	345
S. pneumoniae (-)	1	7	8	51	50	101
Total	15	24	39	182	264	446

Chapter 8 – Summary

Streptococcus pneumoniae is the most important cause of respiratory bacterial disease globally. This dissertation examined whether and how other respiratory pathogens, including the influenza virus, *Haemophilus influenzae,* and *Staphylococcus aureus* interact with *S. pneumoniae* to influence its colonization and pathogenesis.

The first two studies investigated the interaction of *S. pneumoniae* and influenza virus in severe influenza pandemics. In the first study, I used a systematic review of antemortem cultures from normally sterile sites and a meta-analysis of bacterial vaccine studies to show that the majority of pneumonias and deaths in the 1918 pandemic were likely due to secondary pneumococcal pneumonia. It was also found that although bacterial vaccines could not prevent influenza infection, the use of whole-cell pneumococcal vaccines was associated with case – fatality reduction of 42% in civilians and 70% in the military among influenza patients.

Previous epidemiologic and animal studies suggest that underlying mechanisms of the interaction between influenza virus and *S. pneumoniae* include the possibilities that influenza infection can: 1) increase the risk of acquisition and colonization of the pneumococcus; 2) increase the aerosolization of the colonizing pneumococci, facilitating their spread to others; and, 3) increase host susceptibility to pneumococcal pneumonia. In my second study, I developed a mathematical co-infection model taking into account these three possible interaction effects of influenza virus and *S. pneumoniae* to predict morbidity and mortality in a future pandemic with an influenza virus having the virulence and transmissibility of that of 1918. The model predicts

that the prevalence of pneumococcal colonization would greatly influence the severity of a "1918-like" influenza pandemic. It also predicts that such a pandemic will result in many fewer deaths in current developed countries than in 1918 simply due to the decline in pneumococcal carriage and the herd immunity effect provided by the widespread use of pneumococcal conjugate vaccines given to infants, even without other interventions such as influenza vaccines, antivirals, antibiotics or access to intensive care. Antibiotic treatment of patients with secondary pneumonia can greatly reduce mortality. Although antibiotic prophylaxis can reduce the incidence of pneumococcal pneumonia in a severe influenza pandemic, it will be less useful in high-resource settings because the number needed to prophylaxis is too high.

In the third study, I examined the interaction between *S. pneumoniae* and two other common respiratory bacteria, *Haemophilus influenzae,* and *Staphylococcus aureus* in the nasopharynx using nasopharyngeal samples from a longitudinal study of Peruvian children. Real-time PCR and culture were performed to determine and quantify colonization by these three bacteria. It was found that children colonized by *S. pneumoniae* are more likely to be colonized by *H. influenzae* but less likely to be colonized with *S. aureus*, suggesting that *S. pneumoniae* and *H. influenzae* interact in a way that results in greater colonization by both while *S. pneumoniae* and *S. aureus* interfere with each other in the nasopharynx.

The results from my three dissertation studies indicate that influenza virus, *H. influenzae* and *S. aureus* can interact with *S. pneumoniae* and influence colonization with, transmission of, and disease due to *S. pneumoniae*. These results have important public health implications. Understanding the joint effects of *S. pneumoniae* and

influenza virus helps us to postulate a more conservative upper bound for the disease burden of a future 1918 – like influenza pandemic because previous modeling studies, without considering the declining pneumococcal prevalence, could greatly overestimate the disease burden. Additionally, since antiviral interventions are more expensive and not affordable in developing countries, the results suggest that antibacterial interventions may be cost-effective alternatives in those countries. The results also suggest that we need to consider the effect of bacterial interaction and monitor bacterial flora when introducing a new vaccine or antimicrobial drug which targets a specific pathogen because other pathogens may be unexpectedly and unwantedly altered.

S. pneumoniae, like many other pathogens, is often studied individually, although it co-exists or competes with many other microorganisms and may involve complex microbial interaction in the human body. Although infections can be caused by a single pathogen, many infections probably result from a combination of different pathogens which affect each other in transmission and pathogenesis. It is important to understand the interaction between pathogens to design better treatment and preventive measures and predict possible repercussions of interventions designed to thwart only a single pathogen.

Appendix

Bacterial Pathogens and Mortality during the 1918 Influenza Pandemic

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A review of recut lung tissue specimens and published autopsy series from the 1918 influenza pandemic suggest that most deaths resulted from secondary bacterial pneumonia,[1] consistent with our preliminary analysis of ante-mortem blood cultures and the time to death of patients.[2] We review here sterile site ante-mortem cultures from an extensive archive in all languages of 1918 pandemic papers (http://www3.niaid.nih.gov/topics/Flu/1918/bibliography.htm). Bacteria were rarely recovered in blood from influenza patients without pneumonia (0.2%), but were commonly isolated from influenza – associated pneumonia cases (15.7%; range 1.6%–50%) and from fatal cases (40%) (Table 1). Moreover, 79.6% of pleural and lung cultures from pneumonia patients yielded bacteria (range 56.7%–100%). Pneumonia studies with lower positive culture rates might have taken cultures early, over-diagnosed pneumonia or had problems culturing fastidious organisms. *Streptococcus pneumoniae* and hemolytic streptococci (probably *Streptococcus pyogenes*) comprised 70.6% and 28.0%% of positive blood, pleural and lung cultures respectively, while *Staphylococcus aureus* was less common (0.8%).

The insensitivity of blood culture to identify pneumococcal pneumonia (~3% in children and ~20% in adults) and the high percentage of positive lung cultures,[3] suggest that bacterial infections, especially pneumococcal infections, were the major cause of influenza – associated pneumonia and death in 1918-1919. Military and civilian populations had similar proportions of positive bacterial cultures among pneumonia cases. The pneumococcal serotype distribution shifted to less invasive serotypes compared to the pre-1918 period, suggesting that the 1918 influenza virus increased host susceptibility to less invasive pneumococci.[2]

Children receiving pneumococcal conjugate vaccine (PCV) in a double blind randomized trial had a 45% reduction in seasonal influenza – related pneumonia

hospitalization.[4] Recent use of PCV in developed countries and availability of antibiotics since the 1940's may have reduced mortality associated with influenza pandemics. Consistent with our results, a recent CDC report shows that 43% of pediatric deaths in the USA associated with 2009 pandemic influenza A (H1N1) had positive cultures from sterile sites or post-mortem lung biopsy, with staphylococci predominant.[5] The staphylococcal predominance may reflect its resistance to community antibiotic use and a higher probability of culture post mortem or after antibiotic therapy.

A larger burden of pneumonia – related pandemic influenza mortality could occur in developing countries during the current pandemic with high rates of pneumococcal carriage in both adults and children, and limited PCV and antibiotic access. These data suggest that pneumococcal vaccination and antibiotic therapy may be important to reduce influenza – associated pneumonia mortality.

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Appendix Table 1: Results of ante-mortem blood cultures and pleural effusions / lung cultures from the 1918 pandemic, stratified by disease status at the time of culture.

Blood cultures from influenza patients without pneumonia, cases with pneumonia, and influenzal pneumonia cases with subsequently fatal outcome									
Disease status	Population	No. studies included	No. cases	No. positive cultures	% positive cultures	No. positive pneumococci	No. positive hemolytic streptococci	No. positive *S. aureus*	No. other bacteria or undetermined
Without pneumonia	Military	5	323	0	0%	0	0	0	0
	Civilian	5	86	1	1.2%	0	1	0	0
	Total	10	409	1	0.2%	0	1	0	0
With pneumonia	Military	16	2042	290	14.20%	238	49	2	3
	Civilian	8	323	81	25.70%	36	32	2	11
	Total	24	2365	371	15.7%	274	81	4	14
Fatal pneumonia	Civilian	3	45	18	40%	8	1	0	9
Cultures from pleural effusions and lung punctures from influenza patients with pneumonia									
Disease status	Population	No. studies included	No. cases	No. positive cultures	% positive cultures	No. positive pneumococci	No. positive hemolytic streptococci	No. positive *S. aureus*	No. other bacteria or undetermined
With pneumonia	Military	5	224	182	81.3%	140	55	0	10
	Civilian	2	61	45	73.8%	9	31	1	5
	Total	7	285	227	79.6%	149	86	1	15

*Results for individual studies are given in the Supplementary Appendix, available with the full text of this article at NEJM.org