Molecular Epidemiology of *Bordetella pertussis*

By

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Dedication

To my wonderful husband, Paul. We did it! This would not be possible without your never-ending encouragement and support. You took on so much to allow me to focus on achieving my goals.

To my mother. You are the definition of strength. I guess sitting on the steps with me every day to go through every one of the papers in my backpack paid off.

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List of Abbreviations

µm	Microliters
µm	Micrometers
µSec	Microsecond
aP	Acellular pertussis
C	Celsius
CDC	Centers for Disease Control and Prevention
CO2	Carbon dioxide
DNA	Deoxyribonucleic acid
DTaP	Diphtheria, tetanus, acellular pertussis vaccine
DTP	Diphtheria, tetanus, pertussis vaccine
EPI	Expanded Programme on Immunization
FDA	Food and Drug Administration
FHA	Filamentous hemagglutinin
FIM2	Fimbrial agglutinogen 2
FIM3	Fimbrial agglutinogen 3
g	Gravity
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IRB	Institutional Review Board
MDCH	Michigan Department of Community Health
min	Minute

mm	Millimeters
nm	Nanometers
NNDSS	National Notifiable Disease Surveillance System
NP	Nasopharyngeal
OD	Optical density
PBS	Phosphate buffer solution
pH	Per hydrogen
PRN	Pertactin
PT	Pertussis toxin
ptxP	Pertussis toxin promoter
SNP	Single nucleotide polymorphism
Tdap	Tetanus, diphtheria, acellular pertussis booster vaccine
US	United States
WA	Washington

Abstract

Bordetella pertussis is the causative agent of whooping cough, a highly contagious infection of the upper respiratory tract that can lead to particularly severe disease in infants and young children, including death. A whole cell vaccine was introduced in the 1940s leading to a rapid decline in the number of cases; however adverse events from the vaccine led to the development and release of a safer acellular vaccine in the 1990s. Since the introduction of the acellular vaccine, pertussis cases began to rise. The past few years have seen a particularly large resurgence in cases; in 2012 the number of reported cases in the United States was the highest since 1955. Reasons for this resurgence are not entirely clear. As a large proportion of cases are in fully vaccinated individuals, we hypothesized that interactions with host microbiota through coaggregation interactions may play a role in who gets infected. We further hypothesized that vaccination selected for *B. pertussis* strains without the antigens included in the vaccine.

To explore the ability of *B. pertussis* to coaggregate with common commensals of the nasopharynx we developed a high-throughput method for its detection. I applied this method to screen for coaggregation between 10 *B. pertussis* strains with 20 nasopharygeal commensal strains. We also used whole genome sequencing and phylogenetic analysis of 100 *B. pertussis* isolates randomly selected from 8 vaccination time periods to test whether vaccination produced a bottleneck in the *B. pertussis* genome.

There was apparent coaggregation between *B. pertussis* and strains of *H. influenzae, P. aeriginosa, S. aureus*, *S. pyogenes*, and *S. pneumoniae;* however visual examination using the FlowCam™ runs and confocal microscopy suggested induction of

autoaggregation in *B. pertussis* by *S. aureus* and *P. aeruginosa*, and no interaction between *B. pertussis* and the other strains. By inducing autoaggregation in *B. pertussis*, *S. aureus* and *P. aeriginosa* may be able to prevent *B. pertussis* from colonizing the host. Analysis of the genetic sequence data suggests that pre-vaccine era isolates are distinct from post-vaccination strains ($p<0.0001$) and that *B. pertussis* underwent a bottleneck.

Chapter 1. Introduction

1.1 Bordetella pertussis: Clinical Symptoms and Changing Epidemiology

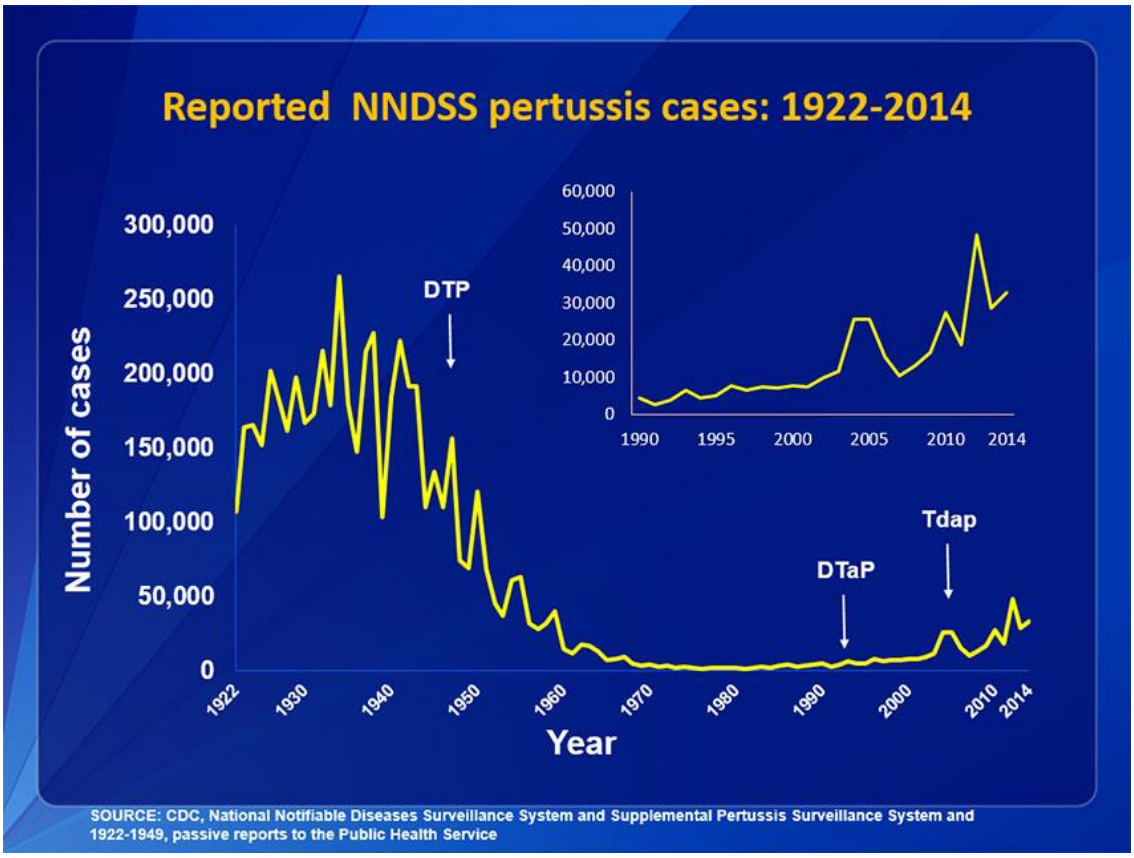
Pertussis infection (whooping cough) is caused by the bacterium *Bordetella pertussis,* a Gram negative coccobacillus that attaches to the ciliated epithelial cells of the nasopharynx (Melvin et al. 2014). Pertussis is a highly contagious infection of the upper respiratory tract and is characterized by severe episodes of paroxysmal coughing followed by an inspiratory whoop (Council of State and Territorial Epidemiologists 2010). Pertussis is clinically defined as a cough illness that lasts more than two weeks and includes one or more of the following symptoms: paroxysms of coughing, inspiratory whoop, posttussive vomiting, and – for infants under one year - apnea (Centers for Disease Control and Prevention 2016). The natural history of clinical pertussis has three separate stages. The first stage, catarrhal, typically begins with general upper respiratory tract symptoms (rhinorrhea and cough). One to two weeks later the paroxysmal stage begins and includes severe coughing episodes typically ending in an inspiratory whoop. The final stage, the convalescent stage, occurs 2-4 weeks later and is characterized by improvement of symptoms, although a cough can remain for one to three months (Halperin SA 2012). Individuals of all ages can be infected, but adults and adolescents typically have milder cases or may be asymptomatic, whereas infection in infants can lead to severe complications and can be fatal (Halperin SA 2012).

A whole cell pertussis, diphtheria, and tetanus combination vaccine (DTP) was introduced in the United States in the mid-1940's (Centers for Disease Control and Prevention 2016). Since widespread vaccination began in North America, the number of

reported cases has decreased by over 90% (Halperin SA 2012). Due to concerns over reactogenicity with the whole cell vaccine, an acellular vaccine (DTaP) was introduced in the United States and licensed for the fourth and fifth doses of the childhood vaccinations series in 1991 and for the entire five dose series by 1997 (Schmidtke et al. 2012). An acellular adolescent and adult booster (Tdap) was recommended in 2005.

In the pre-vaccine era, pertussis infection was common causing an average of 178,000 reported cases per year and affecting mostly children aged 1-9 years (Figure 1) (Centers for Disease Control and Prevention 2016; Melvin et al. 2014). Following the introduction of the whole cell vaccine in the 1940s, the number of reported cases fell to approximately 17,000 annually during 1950-1990 (Figure 1) (Centers for Disease Control and Prevention 2016). A resurgence in reported cases coincided with the introduction of the acellular vaccine in 1991 and by 2012, the number of reported cases increased to nearly 42,000 per year (Figure 1) (Centers for Disease Control and Prevention 2016). The epidemiology since the resurgence has changed substantially: with pertussis infection being more common in infants (<1 years) and older children (ages 9-19) (Melvin et al. 2014). Further, cases in older children and adults often occur in fully vaccinated individuals. During a *B. pertussis* outbreak in California during 2010, 79% of the infected children ages 7-10 were fully vaccinated (Winter et al. 2012). Population data from 1996-2010 in Norway found 90% of the cases among those 14 and older were fully vaccinated (Lavine et al. 2010). Although the whole cell vaccine appears to provide a longer duration of immunity, both the whole cell vaccine and the acellular vaccine fail to induce sterilizing immunity (Locht 2016; Warfel et al. 2014; Witt et al. 2012).

Figure 1.1 Reported National Notifiable Disease Surveillance System (NNDSS) reported pertussis cases: 1922-2014



Reasons for this resurgence are not entirely clear. Some current hypotheses include: waning immunity over time following vaccination or infection (Tan et al. 2005; Wearing & Rohani 2009), antigenic divergence of circulating strains in response to selective pressures from vaccines (Mooi et al. 2001), decreased vaccination rates (Omer et al. 2009), variable vaccine efficacy (Crowcroft & Pebody 2006; Ntezayabo et al. 2003), age-structured contact patterns (Rohani et al. 2010), and acellular vaccines failing to prevent transmission by protecting against disease but not infection leading to an increase in asymptomatic carriage (Warfel et al. 2014). Lending further support to acellular vaccines resulting in asymptomatic carriage, in a secondary analysis of an acellular vaccine efficacy trial, researchers found that while the vaccine did prevent the disease, it did not protect against infection or colonization (Storsaeter et al. 1990). In

addition, an antibody study in a vaccinated cohort of healthy one-year olds found 5-10% had evidence of recent *B. pertussis* infection although few clinical cases were recognized in the community at the time (von Linstow et al. 2010).

1.2 Pertussis Vaccine and Antigenic Divergence

The current acellular pertussis vaccines contain five *B*. *pertussis* components: pertussis toxin (*ptxP* and *ptxS1*), filamentous hemagglutinin (*fha*), fimbrial agglutinogens 2 and 3 (*fim*2 and *fim*3), and pertactin (*prn*) (Decker & Edwards 2000) (Figure 1.2). The two main manufacturers of the acellular vaccine used in the United States are GlaxoSmithKline and Sanofi Pasteur. The strain used for the manufacture of the GlaxoSmithKline vaccine in the United States is Tohama 1, a strain first isolated in Japan, which has an allelic variant profile of *prn (1)-ptxP(1)-ptxS1B-fim2(1)-fim3(A)* (Schmidtke et al. 2012; Bottero et al. 2007). The Sanofi Pasteur acellular pertussis vaccine is manufactured from *B. pertussis* strain 10536 (*prn(1)-ptxP(1)-ptxS1D-fim2(1)-fim3(A))*(Bottero et al. 2012) obtained from the Michigan Department of Health (Grabenstein 2012).

Since the introduction of the acellular vaccine, the most common circulating strains in the United States have diverged away from vaccine strain alleles (Table 1.2). For example, since 1991 the *ptxP3* allele – associated with increased toxin production - has become the dominant *ptxP* allele in the United States replacing the *ptxP1* allele found in both vaccine strains (Schmidtke et al. 2012; Mooi et al. 2009). Antigenic divergence of circulating strains in response to selective pressures from vaccines has been observed worldwide (Mooi et al. 2001; Mooi et al. 2009; Bart et al. 2014; Bart et al. 2010). Antigenic divergence of circulating strains away from vaccine strains might render the vaccine less effective resulting in an increase in cases. This has already been observed in the Netherlands, where the shift to *ptxP3* toxin was associated with an increase in pertussis infection notifications (Mooi et al. 2009).

Figure 1.2. Acellular vaccine component locations on *B. pertussis* cellular surface

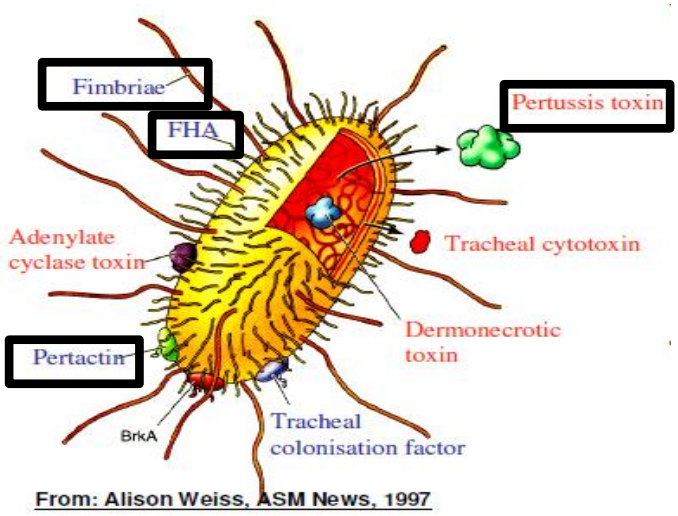


Table 1.1 Genotype of vaccine strains, number of circulating alleles, and predominate allele in the United States

		Acellular Vaccine Components					**Sources**
		prn	*ptxP*	*ptxS1*	*fim2*	*fim3*	
Vaccine Strains	Tohama 1 genotype	1	1	B	1	A	(Schmidtke et al. 2012; Bottero et al. 2012)
	10536 genotype	1	1	D	1	A	(Bottero et al. 2012; Grabenstein 2012)
Number of circulating alleles		2	11	6	2	4	(Kallonen & He 2009; Mooi et al. 2009; Locht 2007)
Predominate allele in US		2	3	A		B	(Schmidtke et al. 2012; Cassiday et al. 2000)

1.3 Asymptomatic Carriage

Individuals with unrecognized pertussis infections can serve as reservoirs for transmission in hospital settings and within families (Conover & Sloan 2010). While little is known about the prevalence of asymptomatic carriage in the absence of vaccination, neither the acellular nor whole cell vaccines were able to prevent colonization and

asymptomatic infection as well as natural immunity from a previous infection (Warfel et al. 2014). Using a baboon model for *B. pertussis* infection, Warfel et al. and found that while vaccination with acellular pertussis (ap) protected against severe symptoms, it did not protect against colonization (Warfel et al. 2014). Importantly, vaccinated baboons did not clear the infection any faster than naïve animals and could readily transmit *B. pertussis* to their unvaccinated contacts (Warfel et al. 2014). By comparison, the whole cell vaccine induced more rapid clearance in both aP-vaccinated and naïve animals (Warfel et al. 2014). Natural infection completely prevented colonization upon secondary infection (Warfel et al. 2014). Warfel et al. propose that asymptomatic infection and subsequent transmission by individuals vaccinated with the acellular vaccine could, in part, explain the increase in observed *B. pertussis* incidence (Warfel et al. 2014). In a study examining the household contacts of 16 infants hospitalized with *B. pertussis* infection, parents and siblings, nearly half of which were asymptomatic, were found to serve as an important source of infection (Raymond et al. 2007).

1.4 Nasopharyngeal Microbiota may Mediate Risk of Pertussis Infection

The human microbiota is comprised of the ecological community of commensal, symbiotic, and pathogenic microorganisms found in and on the human body (Lederberg 2001). These organisms can protect the host from potentially invading pathogens through both microorganism-mediated direct inhibition and indirectly by enhancing host immunity (Buffie & Pamer 2013). There is some evidence that host microbiota can influence the ability of *B. pertussis* to colonize. Using a murine model Weyrich et al. determined that delivering a broad-spectrum antibiotic treatment before *B. pertussis* inoculation reduced the infectious dose from 10,000 CFU to less than 100 CFU and that reintroduction of a single *Staphylococcus* or *Klebsiella* species was sufficient to inhibit *B. pertussis* colonization in antibiotic-treated mice (Weyrich et al. 2014). These results show that host microbiota can influence host specificity and prevent colonization by *B. pertussis* in mice (Weyrich et al. 2014). We hypothesize that interactions with host microbiota are also an important determinant in the ability of *B. pertussis* to colonize

humans. With a large number of infections occurring in fully vaccinated individuals, clearly other factors contribute to who becomes infected.

B. pertussis might interact with the host microbiota through several mechanisms. *Bordetella* species contain many mechanisms for interbacterial competition including a type IV secretion system for secretion of pertussis toxin, bacteriophages, and potentially contact-dependent growth inhibition (Weyrich et al. 2014). Coaggregation, the highly specific recognition of surface molecules between two genetically distinct bacterial cell types, is common among oral bacteria and may be another mechanism through which *B. pertussis* interacts with the host microbiota (Hughes et al. 1988). Coaggregating organisms and multi-species biofilms have many advantages compared to planktonic cells such as resistance to antimicrobials, the host immune system, and shear forces, and are able to share nutrients (Rickard et al. 2003; Mishra & Parise 2005; Watnick & Kolter 2000).

The ability to coaggregate is important for the development of multispecies biofilms, which may allow *B. pertussis* to persist in the nasopharynx of the host. Biofilms consisting solely of *B. pertussis* are well documented, but whether it is able to jointly form biofilms with other species, particularly nasopharyngeal commensals in human hosts, is unstudied (Arnal et al. 2015; Conover & Sloan 2010; Serra et al. 2011; Mishra & Parise 2005; Conover et al. 2012). Coaggregation interactions are likely important for adherence and colonization of bacteria to a variety of host surfaces (Kolenbrander 1988). It may be that the presence of certain nasopharyngeal commensals is necessary for *B. pertussis* to attach or the presence of certain commensals may actively prevent *B. pertussis* attachment through antagonistic coaggregation interactions.

With a large proportion of cases occurring in fully vaccinated individuals, interaction with host microbiota may play a deciding factor in who gets infected among vaccinated individuals. If *B. pertussis* is able to coaggregate with nasopharyngeal commensals, it might lead to persistence in the nasopharynx. Persistence in the nasopharynx may result in asymptomatic carriage which is common in adolescents and

adults (Halperin SA 2012). Asymptomatic carriage also is believed to be increasing due to the acellular vaccine preventing disease, but failing to prevent transmission and may be a key component of transmission dynamics (Warfel et al. 2014; Althouse & Scarpino 2015). Lastly, the acellular vaccine is targeted against only a few *B. pertussis* antigens and may result in selection of non-vaccine strains resulting in vaccine failure. Antigenic divergence with respect to several of the vaccine components has already occurred (Mooi et al. 2001; Bart et al. 2014; Borisova et al. 2007; Schmidtke et al. 2012).

1.5 Dissertation Aims

The overall goal of this dissertation is to evaluate some of the hypothesized mechanisms leading to the resurgence of pertussis in the United States. Chapters 2 and 3 evaluate the role of coaggregation. To do so, we first developed a high-throughput, quantitative method to screen for coaggregation among bacterial species (Chapter 2). The method uses optical density in a 96-well plate format to simultaneously assess for coaggregation among multiple replicates of a large number of test crosses and then confirms these results with a FlowCam™ device for measuring size of particles over time in a flowing environment, and confocal microscopy. In Chapter 3 we apply this high-throughput screening method to assess for coaggregation between *B. pertussis* strains and common commensals of the nasopharynx. In Chapter 4, we screen nasopharygeal swab samples taken from 519 healthy adults from 115 households in Vinh Thanh, Vietnam to estimate of the rate of asymptomatic carriage of *B. pertussis*. We also screen this collection for co-occurrence of *B. pertussis* with the nasopharyngeal commensals that we found to interact with *B. pertussis* in Chapter 3. As noted earlier, screening for commensals identified a problem with this collection and raised serious questions about the validity of the resulting estimates. Chapter 5 describes results of whole genome sequencing and phylogenetic analysis to identify whether vaccination resulted in a genetic bottleneck. The last chapter provides a general summary of the knowledge and skills gained through this dissertation work, including a reflection on the challenges of inherent variability of coaggregation, finding and obtaining a large

collection of isolates for sequencing, using archival collections, and of learning the very diverse set of skills necessary to conduct these various studies.

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Chapter 2. High Throughput Quantitative Method for Assessing Coaggregation among Oral Bacterial Species

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2.1 Significance and Impact of the Study

Coaggregation between bacterial species is integral to multi-species biofilm development. Difficulties in rapidly and reproducibly identifying and quantifying coaggregation have limited mechanistic studies. This paper demonstrates two complementary quantitative methods to screen for coaggregation. The first approach uses a microplate-based high-throughput approach and the other uses a FlowCam™ device. The microplate-based approach enables rapid detection of coaggregation between candidate coaggregating pairs of strains simultaneously while controlling for variation between replicates. The FlowCam™ approach allows for in-depth analysis of the rates of coaggregation and size of aggregates formed.

2.2 Abstract

This paper describes a high throughput method that relies upon a microplate reader to score coaggregation 60 minutes post mixing, and use of a high-speed real-time imaging technology to describe the rate of coaggregation over time. The results of visual, microplate, and FlowCam™ aggregation scores for oral bacteria *Streptococcus*

gordonii, Streptococcus oralis, and *Actinomyces oris,* whose ability to coaggregate are well characterized, are compared. Following mixing of all possible pairs, the top fraction of the supernatant was added to a microplate to quantify cell-density. Pairs were also passed through a flow cell within a FlowCam™ to quantify the rate of coaggregation of each pair. Results from both the microplate and FlowCam™ approaches correlated with corresponding visual coaggregation scores and microscopic observations. The microplate-based assay enables high throughput screening, whereas the FlowCam™-based assay validates and quantifies the extent that autoaggregation and coaggregation occur. Together these assays open the door for future in-depth studies of autoaggregation and coaggregation among large panels of test strains.

2.3 Introduction

Coaggregation is defined as the highly specific recognition and adherence of different species of bacteria with each other (Kolenbrander 1988; Rickard et al. 2003). Coaggregation typically occurs as a consequence of the expression of protein adhesins on the cell-surface of one bacterial species, and complementary polysaccharide-containing receptors expressed on the surface of the other bacterial species (Kolenbrander 1988; Rickard et al. 2003).

Coaggregation interactions are important for the development of multi-species biofilms (e.g. dental plaque). It contributes to biofilm development via at least two mechanisms: i) free floating planktonic cells of one species specifically recognize cells of another species type and co-adhere to the developing biofilm, and/or ii) bacterial cells of a planktonic species recognize and coaggregate with cells of another species within an established biofilm community (Rickard et al. 2003; Kolenbrander et al. 2010). It is likely that these interactions are important for adherence and colonization of bacteria to a variety of biotic and abiotic surfaces, and provide selective advantages against non-coaggregated bacterial species contained within a biofilm (Kolenbrander et al. 1990; Busscher & Van Der Mei 1995; Burmølle et al. 2006; Kolenbrander et al. 2010).

The oral microorganisms *Streptococcus oralis*, *Streptococcus gordonii* and *Actinomyces oris* strongly coaggregate and are considered early colonizers in the process of dental plaque formation (Kolenbrander 2000). Early colonizers anchor the biofilm to the substratum surface and thereby contribute to recalcitrance of the biofilm to removal (Busscher & Van Der Mei 1995). For example, the presence of *A. oris* greatly reduces the ability of the *S. oralis* to be removed by shear force when compared with direct attachment of *S. oralis* to the pellicle (proteinaceous conditioning film formed on the tooth surface) (Busscher & Van Der Mei 1995). Furthermore, early colonizers provide a foundation for other species to adhere, forming a mature biofilm community (Busscher & Van Der Mei 1995). Through autoaggregation (aggregation within a single species) or coaggregation, organisms can individually and collectively obtain increased resistance towards antimicrobials and shear forces, communicate via cell-cell signaling, and share nutrients (Kinder & Holt 1994; Watnick & Kolter 2000; Rickard et al. 2003). Research using model dental plaque systems has shown nutritionally mutualistic relationships occurring between coaggregating organisms (Bradshaw et al. 1994; Palmer et al. 2001). For example, *A. oris* and *S. oralis* displayed limited to no growth when grown in monoculture with saliva as the nutrient source, but thrived when allowed to coaggregate (Palmer et al. 2001).

Traditionally, coaggregation is assessed using a visual scoring system based on the size of the coaggregates and turbidity of the supernatant fluid (Cisar et al. 1979; Gilbert et al. 2002; Vornhagen et al. 2013). However, as visual scoring is only semi-quantitative, it is subject to inconsistency and bias in scoring (Busscher & Van Der Mei 1995). Another method, measuring the percent change in optical density, provides a quantitative assessment and greatly improves reliability and reproducibility. However, current methods are not amenable to screening of larger numbers of samples simultaneously (Ikegami et al. 2004; Ledder et al. 2008; Nagaoka et al. 2008; Arzmi et al. 2015) and these technological insufficiencies have limited the in depth study of coaggregation (Katharios-Lanwermeyer et al. 2014). The ability to include multiple

replicates in a single experiment is highly desirable, as there may be strain variations in coaggregation requiring multiple crosses to determine if the observed coaggregation occurs generally between two species. Furthermore, because bacterial coaggregation is sensitive to a variety of influences including presence of chelating agents (Taweechaisupapong & Doyle 2000), temperature (Postollec et al. 2005), growth media, and pH (Min et al. 2010), and growth phase of the batch culture cells (Rickard et al. 2000), high throughput methods would be highly desirable to improve reproducibility of results.

Cognizant of these issues, we developed a quantitative method for high throughput screening for coaggregation among bacterial species. Our high throughput method allows for simultaneous analysis of multiple replicates so that experimental variation is reduced and possible subjective bias is minimized. This method's accuracy as a preliminary screening tool was validated using confocal microcopy and a recently developed approach using FlowCam™ technology (Segaloff et al. 2014). A FlowCam™ is a dynamic imaging particle analyzer that examines fluid through a microscope and captures images of the particles as they are pumped through a flow cell via a computer controlled syringe pump. Specifically, a FlowCam™ characterizes the particles using a variety of measurements such as area-based diameter. It has been used in a variety of different industries including aquatic research, algae technology, waste management, pharmaceutical, and oil and gases for purposes such as monitoring algae for biofuels, quantifying protein aggregates in pharmaceuticals, and analyzing drilling products (http://www.fluidimaging.com/). In practice, the high throughput method can be used to screen a large panel of test strains for potential coaggregation. Ideally, strains giving a positive result with the high throughput method would then be tested further using either confocal microscopy or FlowCam™.

Without a high throughput, quantitative method for assessing coaggregation, it is difficult to explore the importance of coaggregation for the development of biofilms. A better understanding of coaggregation can provide a deeper knowledge of how

organisms interact and biofilms form. For example, the presence of biofilms can result in the corrosion of sewer pipes. An improved understanding of if and how organisms coaggregate in these biofilms could help in developing strategies to reduce the detrimental effects of species in biofilms on pipe surfaces (Jensen et al. 2016). A high throughput screening method would also be of interest to the dental research community as identifying coaggregation between oral bacterial species (beyond those already known) could be an important step in developing a fuller understanding of dental plaque development (Kolenbrander et al. 1990). Coaggregation may also be an important mechanism through which pathogens interact with the host microbiota. Younes et al. demonstrated a rapid anti-pathogen effect of probiotic lactobacilli with toxic shock syndrome toxin 1-producing *Staphylococcus aureus* strains as a result of coaggregation (Younes et al. 2012). High throughput studies of coaggregation between organisms could be useful in identifying probiotic species.

2.4 Results and Discussion:

2.4.1 High throughput quantitative method increases validity and reliability of results

Three strains of oral bacteria were used: *Streptococcus oralis 34, Streptococcus gordonii DL1*, and *Actinomyces oris* T14V. *S. gordonii* is a primary colonizer in dental plaques and was previously found to coaggregate with both *A. oris* T14V and *S. oralis* 34 (Cisar et al. 1979). All possible pairwise combinations of these three strains were tested, resulting in six potential coaggregative or autoaggregative pairings. Coaggregation was first assessed in a low throughput format and scored using the visual scoring system developed by Cisar and colleagues (Cisar et al. 1979). As shown in Figure 1A, the maximum visual coaggregation (score = 4) is easily distinguished from no coaggregation (score = 0), but visual intermediate scoring is more subjective and as a consequence is less reproducible.

Using the microplate-based approach with OD 620 nm, it was determined that *S. gordonii* DL1 + *A. oris* T14V, *S. oralis* 34 + *A. oris* T14V, and *S. oralis* 34+ *S. gordonii* DL1 all strongly coaggregated (Figure 1B). No autoaggregation was detected for *S. oralis* 34 or *S. gordonii* DL1. Some autoaggregation was observed for *A. oris* T14V, although the OD did not differ significantly from that of non-autoaggregating strains.

Within each micro-plate run, pairs were assayed in triplicate. The average coefficient of variation (standard error / mean) for the triplicates was 14% with a median of 9%. The coefficient of variations differed by specific crosses with autoaggregation by *A. oris* being the most variable (ranging from 1-66%). An increase in the coefficient of variation for coaggregation was also observed when *A. oris* was a component of a coaggregating pair.

Many factors contribute to between run variations: bacteria are harvested from separately grown batch cultures, which may differ slightly in length of growth time, exact nutrient content and pH of media, pH of buffers used for washing and re-suspension, number of times strain has been passaged before current growth, and natural biological variation. To minimize these variations, bacteria of a given species were harvested from the same batch culture, although some variation might remain due to the slight variations in timing between admixture and measurement, and true biologic variation in the amount of autoaggregation occurring within each species of a given candidate coaggregative pairing.

Between run and within run variation highlight the need for multiple replicates of each candidate coaggregative pair in addition to replicates of each strain on its own (to assess autoaggregation) within a single run. This is easily possible using the high-throughput 96-well plate method. Quantitatively comparing coaggregative and autoaggregative behavior within a single run also enables more accurate assessments of coaggregation by controlling for any autoaggregation that may occur, and multiple

replicates of all strains and strain pairs allow for construction of confidence intervals around the mean OD value for a given strain or strain pair.

2.4.2 FlowCam™ Technology can measure particle sizes and quantify rate of coaggregation

FlowCam™ technology was used to validate the high-throughput 96-well plate system, providing an in-depth analysis of the rates of coaggregation, and visual and quantitative assessment of the size of aggregates formed (Segaloff et al. 2014). The average particle size per minute increased over time for all three coaggregative pairings (Figure 2). By minute three (two minutes post-mixing) all coaggregating strains experienced a statistically significant increase in average particle area per minute as calculated using area-based diameter. Strong coaggregation occurred when *S. gordonii* and *A. oris* were combined, with particles averaging 212 μm^2 per minute and reaching as large as 3,800 μm^2 in area. The coaggregation between *S. oralis* and *S. gordonii* was not as strong. Particle sizes averaged 122 μm^2 by the final minute of data collection and reached a maximum area of approximately 2,950 μm^2, but many cells did not coaggregate and remained in suspension. This variation in coaggregation between this pair resulted in large confidence intervals around each time point. *S. oralis* and *A. oris* coaggregated strongly with particle sizes averaging 215 μm^2 in area by minute two and reaching sizes as large as 3,180 μm^2. In the autoaggregation assays, the average area of *A. oris* particles (83 μm^2) was significantly larger than those of *S. oralis* (35 μm^2) and *S. gordonii* (51 μm^2), indicating strong autoaggregation in this species. Here, the use of FlowCam™ allowed for quantification of rates of coaggregation and measurement of the particle size associated with coaggregation. Results from FlowCam™ correlated well with the results of the high throughput screen, with coaggregation indicated by increases in particle size over time following the addition of the second organism and autoaggregation indicated by larger particle sizes. FlowCam™ was more useful for detecting autoaggregation than the high throughput screening method on its own, which did not show a statistically significant difference between autoaggregating and

non-autoaggregating strains. These results validate the use of the high throughput method as an initial screening step to be followed up with a more confirmatory assay such as FlowCam™ or confocal microscopy.

2.4.3 Confocal microscopy confirms presence of coaggregation

As a further confirmation, the strains were stained using Syto-9 (green) or Syto-61 (red) nucleic acid stains before crossing them for coaggregation, and then visualized using a confocal microscope (Figure 3). Confocal microscopy images confirmed that *S. oralis* and *S. gordonii* do not autoaggregate (Figure 3, A and C). Visualization of *A. oris* alone confirmed strong autoaggregative behavior (Figure 3B). This was not immediately apparent from initial absorbance readings from the high throughput screening method because autoaggregation was not followed by immediate sedimentation (Figure 1B). This finding is consistent with previous reports. Koop and colleagues showed autoaggregation without associated sedimentation could be missed by spectrophotometry (Koop et al. 1989), highlighting the importance of using a combination of methods for detection. The high-throughput 96-well plate method is most appropriately applied as a screen for potentially coaggregating pairs from a large pool of candidates. Potentially coaggregating pairs should be further evaluated using FlowCam™ or confocal microscopy, ideally both. Moreover, if coaggregation is suspected, autoaggregation should be ruled out.

S. oralis + *S. gordonii* showed moderate coaggregation (Figure 3D) while *S. gordonii* + *A. oris* (Figure 3E) and *S. oralis* + *A. oris* (Figure 3F) showed strong coaggregation. *S. oralis* and *S. gordonii* appeared to coaggregate in a more even manner, suggesting absence of autoaggregation within the two species (Figure 3D), while *S. oralis* + *A. oris* and *S. gordonii* + *A. oris* showed clumps of the same color (red or green) indicating strong autoaggregative behavior by *A. oris* (Figure 3, E and F).

2.5 Summary

Focusing on the interactions between three well-documented coaggregating strains of oral bacteria, we demonstrated that coaggregation can be quantified, and the kinetics of coaggregation and the size of coaggregates formed can be measured. The microplate-based assay enables high throughput screening to identify potentially coaggregating strains, whereas the FlowCam-based assay validates and quantifies the extent that aggregation and coaggregation occur. In the absence of FlowCam™, or in combination with its use, confocal microscopy is a useful tool for confirming the presence or absence of coaggregation following screening of a large panel of strains with the high throughput method. Together these assays open the door for in-depth studies of aggregation and coaggregation among large panels of test strains.

2.6 Materials and Methods:

2.6.1 Growth Conditions

S. oralis 34 and *S. gordonii* DL1 were incubated aerobically with CO2 at 37°C in Schaedler's broth for 24 hours. *A. oris* T14V was incubated aerobically with CO2 at 37°C in Brain Heart Infusion broth for 48 hours. Cells were harvested from batch culture through centrifugation for 12.5 minutes at 3,000 X g and then washed 3 times in coaggregation buffer (Cisar et al. 1979; Rickard et al. 2000). After each centrifugation step, the supernatant was discarded and the pellet was re-suspended in coaggregation buffer. The washed pellets were then suspended in coaggregation buffer to achieve an optical density at 600 nm of 1.5 (±0.1).

2.6.2 Coaggregation and Autoaggregation assays

Coaggregation and autoaggregation were first assessed using a visual coaggregation assay developed by Cisar et al. where visual scores ranged from 0 (no visible aggregates in the suspension) to 4 (large aggregates form and settle leaving a clear supernatant) (Cisar et al. 1979). To assess coaggregation between two strains, 200

µl of each bacterial suspension were combined in a glass culture tube. To assess autoaggregation 400 µl of the single bacterial suspension as placed in a glass culture tube. The culture tubes were then vortexed for ten seconds and rolled gently for an additional 30 seconds (Rickard et al. 2000). Each pair was assayed in triplicate.

Samples were allowed to sit 60 minutes to let coaggregates settle to the bottom of the tube. Any changes in visual coaggregation score following the 60-minute time period were documented. This endpoint was selected after initial testing of the supernatant at 30 minute time intervals over 3 hours; 60 minutes was ideal for good separation between coaggregating and non-coaggregating strains. One hundred microliters of supernatant were removed from each sample and placed in a 96 well flat-bottom plate. Absorbance of the supernatant was measured at 620 (A) using a PerkinElmer 2030 workstation (PerkinElmer Life and Analytical Sciences, Turku, Finland). Mean OD and associated 95% confidence intervals were calculated over all trials for each of the strain pairs and for each strain alone. Because strains were set to the same optical density (1.5) before they were combined, an expected value for the combined pair was calculated based on the average experimental OD of the two components. The mean OD, 95% confidence interval, minimum and maximum for each pair was then compared with the calculated expected value for the pair. Coaggregation was suspected when the expected value was above the upper limit for the 95% confidence interval and was considered when the mean OD was below the expected value. In all cases meeting these criteria, further screening was conducted using FlowCam™ and confocal microscopy.

As an additional visual test of autoaggregation and coaggregation between strains, 300 µl of each bacterial suspension in coaggregation buffer were stained with either Syto-9 (green: Excitation: 488, Emission: 503) or Syto-61 (red: Excitation: 561, Emission: 645) nucleic acid stains (Invitrogen, Carlsbad, CA, USA). Each bacterial suspension was incubated for 30 minutes at room temperature to allow staining of the cells. Cells were washed three times with coaggregation buffer and collected by centrifugation, as

mentioned above. Each bacterial strain was re-suspended in coaggregation buffer and combined for coaggregation. For autoaggregation studies, Syto-9 and Syto-61 stained cells of that species were mixed together. Twenty microliters of each sample were added to the slide and viewed under the microscope. The entire droplet was scanned and a minimum of three representative fields of view were captured for each pair and for each strain alone using Leica confocal laser scanning microscopy (CLSM, SPE, Leica, IL, USA) with a HCX PL APO 40X/0.85 CORR CS objective. Staining and microscopy were repeated twice to ensure consistency of results." Once the microscopy images were taken, the image files were rendered using Imaris (Bitplane, Zurich, Switzerland) computer imaging software.

2.6.3 FlowCam™ Imaging and Quantification of Coaggregation

To confirm the results of our findings, coaggregation was quantified using FlowCam™ technology (Fluid Imaging Technologies, ME, USA). *S. oralis* 34, *S. gordonii* DL1 and *A. oris* T14V were harvested from batch cultures and washed as described above. The washed pellets were re-suspended in coaggregation buffer to achieve an optical density of 1.0 (±0.1) at 600 nm. Prior to loading the cells into the FlowCam™ device, cell suspensions were further diluted 5X in coaggregation buffer to prevent clogging of flow cell. The first species was added to the device and was pumped through until it reached the flow cell. Data collection began once the Olympus UPlanFL N 10X/0.30 objective was successfully focused on the flowing particles. The second species was added to the vessel containing the first species and gently mixed 1 minute after initiation of data collection. FlowCam™ was run for 10 minutes at a flow rate of 0.3 ml/min with images acquired at a rate of 10 frames per second. Flash duration was set to 8 µSec. Particle size was measured using area based diameter (ABD) and a particle filter of 5 to 10000 µm. Visual spreadsheet software was used for data collection. A minimum of 5 FlowCam runs was conducted for each pair with similar results.

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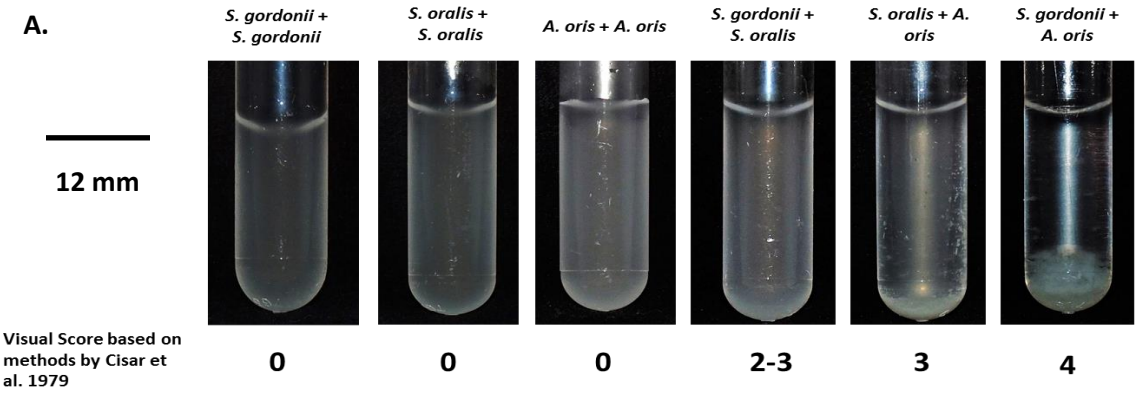
Figure 2.1. (A) Test crosses of pairwise combinations of *S. gordonii*, *S. oralis*, and *A. oris* with associated visual coaggregation score based on methodology by Cisar et al. 1979. (B) Mean Optical Density (620 nm) of supernatant with associated 95% confidence intervals of test crosses.



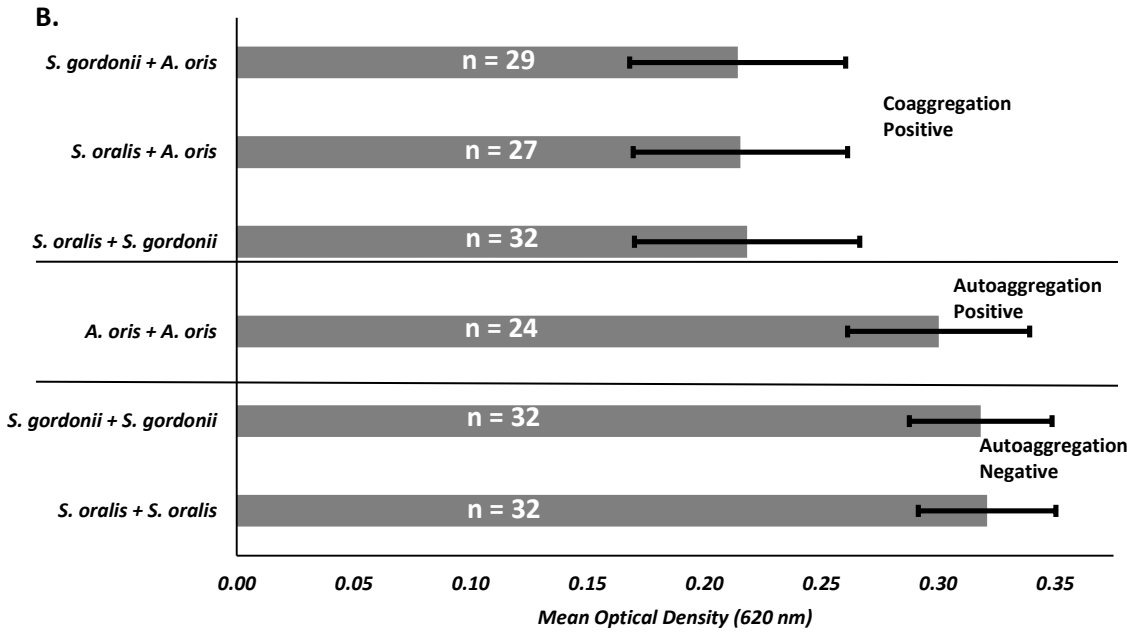


Figure 2.2. Change in average particle area (µm) per minute during 10 minute time period with associated 95% confidence intervals calculated from number of particles scanned per minute for potential (A) coaggregating (*S. gordonii + A. oris, S. oralis + S. gordonii, S. oralis +A. oris*) and (B) autoaggregating (*S. gordonii, A. oris, S. oralis*) pairs as measured with the FlowCam™ device. Calculations were based on an average of 2,009 particles per minute (median = 707, maximum = 5,867, minimum = 24).

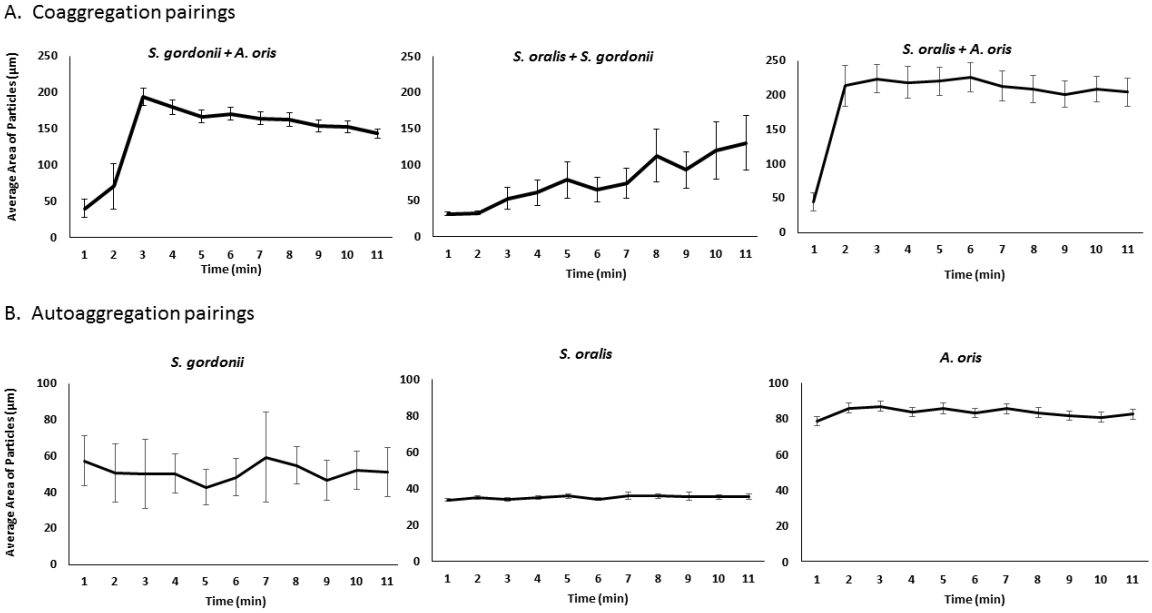
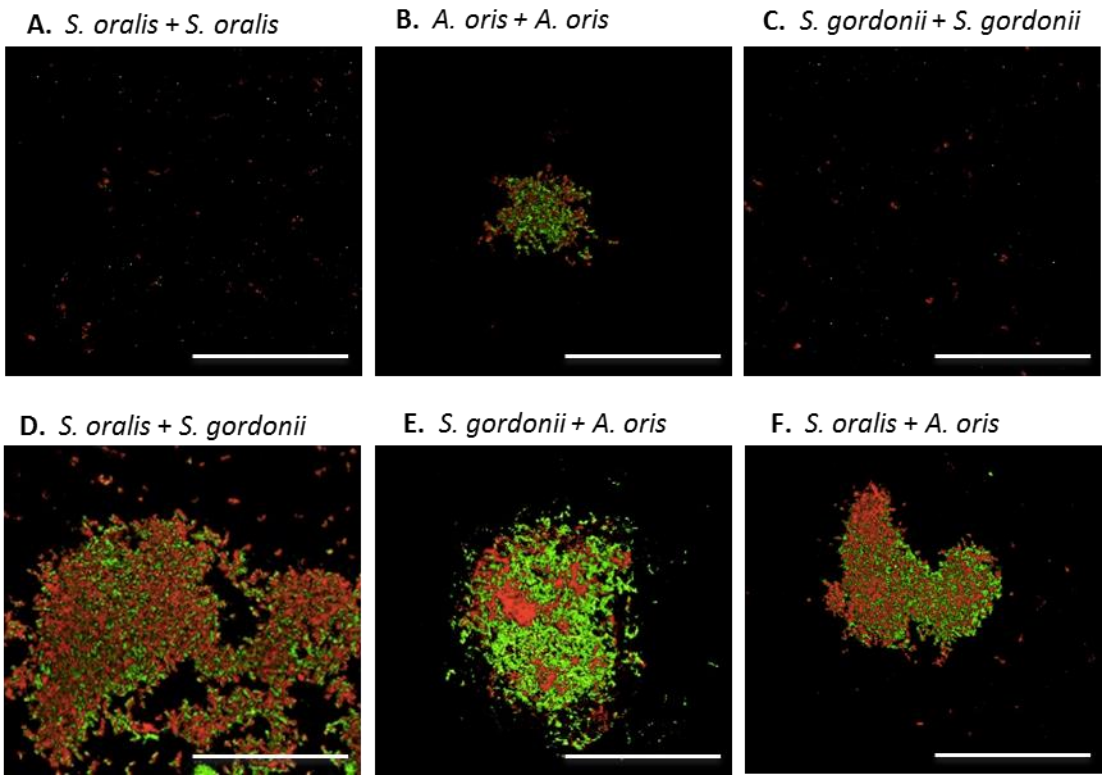


Figure 2.3. Visualization of selection bacterial pairings using confocal microscopy. Confocal microscopy images are represented in the x-y plane. Nucleic acid stains syto-9 (green) and syto-61 (red) was used to detect autoaggregation and coaggregation of oral microbes. Bars represent 40 μM.



Chapter 3. Interactions between *B. pertussis* and other Common Nasopharyngeal Commensals

3.1 Abstract

Bordetella pertussis infection can result in severe morbidity and even mortality in unvaccinated infants and young children, but infection in vaccinated adolescents and adults is often asymptomatic or results in milder symptoms such as a prolonged cough. Asymptomatic carriers are reservoirs for infection and help maintain ongoing transmission. In recent years, the majority of cases have been in fully vaccinated individuals. We hypothesized that coaggregation between *B. pertussis* and members of the host microbiota might be an initial step in *B. pertussis* colonization of the nasopharynx. Whether *B. pertussis* coaggregates is unknown. We tested whether *B. pertussis* coaggregates with common commensals of the human oropharynx (10 strains of *B. pertussis* and 20 nasopharyngeal commensal test strains including *H. influenzae, S. pneumoniae, N. meningitidis*, and *S. aureus.*) using a high-throughput screening method. Strains positive for coaggregation in the high-throughput assay were further evaluated using a FlowCam™ device and confocal microscopy. Using the high-throughput assay there was apparent coaggregation between *B. pertussis* and strains of *H. influenzae, P. aeriginosa, S. aureus*, *S. pyogenes*, and *S. pneumoniae;* however visual examination using the FlowCam™ runs and confocal microscopy suggested induction of autoaggregation in *B. pertussis* by *S. aureus* and *P. aeruginosa*, and no interaction between *B. pertussis* and the other strains. By inducing autoaggregation in *B. pertussis*, *S. aureus* and *P. aeriginosa* may be able to prevent *B. pertussis* from colonizing the host.

3.2 Introduction

3.2.1 Pertussis can be Carried Asymptomatically or with Minimal Symptoms

While pertussis infection can result in severe morbidity and even mortality in unvaccinated infants and young children, infection in vaccinated adolescents and adults is generally milder and results in a prolonged cough (Conover & Sloan 2010). Several studies among vaccinated and unvaccinated adults and adolescents with a cough lasting longer than one week, show high rates of *B. pertussis* in this group*:* from 12-32% (Cherry 1999; Mink et al. 1992; Schmitt-Grohé et al. 1995; Wirsing von König et al. 1995; Wright et al. 1995; Rosenthal et al. 1995; Nennig et al. 1996). Individuals with unrecognized pertussis infections can serve as reservoirs for transmission in hospital settings and within families (Conover & Sloan 2010). In a study of 41 infants under age 4 months and their household contacts, parents and siblings, nearly half of which were asymptomatic, were found to serve as an important source of infection (Raymond et al. 2007).

Only a few studies have estimated the prevalence of asymptomatic carriage. Among a cohort of 242 vaccinated, healthy Danish infants, 5-10% showed IgG and IgA antibodies to pertussis toxin (PT) and filamentous hemagglutinin (FHA) by age 1 although only a few clinical cases were recognized in the community at the time, (von Linstow et al. 2010). This strongly suggests ongoing asymptomatic transmission. Further, among 279 children ages 5-15 years presenting to the Nuffield Department of Primary Care Health Sciences at Oxford University from 2010 to 2012 with a cough illness of two-eight weeks duration, 56 (20% ±5%) had an oral fluid anti-pertussis toxin IgG titre consistent with a recent pertussis infection and of those children, 39 (70%) were fully vaccinated (Wang et al. 2014).

One hypothesized mechanism for *B. pertussis'* ability to persist for long periods in the oropharynx is that *B. pertussis* can form biofilms (Conover & Sloan 2010; Serra et al. 2011; Sloan et al. 2007; Conover et al. 2011) (Arnal et al. 2015; Conover & Sloan 2010; Serra et al. 2011; Mishra & Parise 2005; Conover et al. 2012). Biofilms consisting

solely of *B. pertussis* are well documented, but whether it is able to jointly form biofilms with other species, particularly bacteria found in the nasopharynx of human hosts, is unstudied. When in a biofilm, bacteria are more resistant to antimicrobial agents such as detergents, antibiotics, and reactive oxygen species and are more resistant to clearance by the host's immune system (Conover & Sloan 2010). High levels of resistance to erythromycin and ciprofloxacin among *Bordetella* species in biofilms have been reported (Mishra & Parise 2005) and strains containing Bps, the polysaccharide responsible for biofilm development in *Bordetella* species, took an entire month to be fully cleared from the nose in a murine model (Conover & Sloan 2010). This resistance to clearance by the host's immune system when in biofilm form may allow *B. pertussis* to persist in the nasopharynx of asymptomatic hosts and allow them to serve as reservoirs of infection for unvaccinated infants (Conover & Sloan 2010; Tan et al. 2005).

3.2.2 Oropharyngeal Microbiota may Influence Risk of *B. pertussis* Colonization

Household contacts of pertussis cases vary in risk of infection (including asymptomatic carriage) in ways not entirely explained by vaccination status or previous infection (Raymond et al. 2007; Cherry 1997; Ward et al. 2006). According to the Centers for Disease Control and Prevention, only 9% of the children ages 6 months to 6 years infected during 2015 had not been vaccinated. In a study following the large 2010 pertussis outbreak in Marin County, California, no difference in attack rates were found between fully vaccinated and under- and un-vaccinated children demonstrating that vaccination status is not the primary determinant for who gets infected (Witt et al. 2012).

The majority of those infected are unaware that they are infected and are able to transmit to others (de Melker et al. 2006). Among infants with a known source of infection, more than 66% of the sources of infection were immediate family members (Skoff et al. 2015). In a study of IgG-PT levels (an indicator of recent pertussis infection) in the Netherlands, researchers estimated the incidence of recent infection in the Dutch

population at 6.6%, which was significantly higher than the reported incidence of 0.01% per year. In addition, a study of *B. pertussis* seroprevalence in the Netherlands demonstrated that although increased vaccination has reduced morbidity due to pertussis among children, the infection rate in adults and adolescents has remained unaffected (de Greeff et al. 2010).

One hypothesized mediator of *B. pertussis* colonization is the oropharyngeal microbiota. Some commensals individually or collectively may enhance and others may resist *B. pertussis* colonization. Results from a murine model support this hypothesis. Among mice treated with antibiotics to remove commensals from the nasal cavity, the infectious dose required to obtain *B. pertussis* colonization was significantly reduced: to <100 CFU compared with the 10,000 CFU required before treatment. However, reintroduction of even a single *Staphylococcus* or *Klebsiella* species to the nasal cavity blocked *B. pertussis* colonization following an inoculation of 5,000 CFU of *B. pertussis*, which is greater than ten times the dose required in antibiotic-treated mice (Weyrich et al. 2014). Thus, interactions with host microbiota may mediate the ability of *B. pertussis* to form biofilms and colonize.

Biofilm formation often begins with coaggregation, the highly specific recognition and adherence of two distinct bacterial species (Rickard et al. 2003). Superinfection with other respiratory pathogens such as *H. influenza*, *S. pneumoniae*, and *S. aureus* is common during and after infection with *B. pertussis,* which hints that polymicrobial interactions may be an important aspect of *B. pertussis* infection (Sawal et al. 2009). To gain insight into whether coaggregation might help explain why vaccinated individuals might still asymptomatically carry *B. pertussis*, we assessed the ability of 10 different *B. pertussis* strains to coaggregate with common nasopharyngeal commensals: *S. pneumoniae, S. aureus,* and *H. influenzae, S. pyogenes, P. aeriginosa,* and *N. meningitidis.*

3.3 Methods

3.3.1 Growth Conditions and Strains Used

Haemophilus influenzae (strains 007.2.12, I247, G1322, I316, K29RE2.10) and *Neisseria meningitidis, Streptococcus pyogenes, Staphylococcus aureus* (strains 1263, 25923)*, Pseudomonas aeriginosa, and Streptococcus pneumonia* (serotypes 1, 10, 12F, 16F, 20, 23F, 33, 34, 35B, 7F) strains were incubated aerobically with CO_2 at 37°C in Brain Heart Infusion broth for 24 hours. Cells were harvested from batch culture through centrifugation for 12.5 minutes at 3,000 X g. *B. pertussis* (strains CDC_F657, CDC_F964, CDC_G710, CDC_H971, CDC_I220, CDC_I240, CDC_I369, WA_H174, WA_H930, WA_H962) was grown on Regan-Lowe agar plates at 37°C for 4 days. Coaggregation is known to be highly variable (Levin-Sparenberg et al. 2016), therefore, we also repeated experiments reducing the growth time from 24 to 18 hours for test strains and an increasing growth time from 4 to 5 days for *B. pertussis*.

B. pertussis cells were harvested by swabbing the surface of the agar with a coaggregation buffer-soaked, nylon-tipped swab and suspending the cells in coaggregation buffer before centrifugation for 12.5 minutes at 3,000 X g. All cells were washed 3 times in coaggregation buffer (Cisar et al. 1979; Rickard et al. 2000). After each centrifugation step, the supernatant was discarded and the pellet re-suspended in coaggregation buffer. The washed pellets were then suspended in coaggregation buffer to achieve an optical density at 600 nm of 1.5 (±0.1).

3.3.2 High Throughput Coaggregation and Autoaggregation Assays

To assess coaggregation between two strains, 200 µl of each bacterial suspension were combined in a glass culture tube. Autoaggregation was assessed by examining 400 µl of each sample and continuing with the same protocol. The culture

tubes were vortexed for ten seconds and rolled gently for an additional 30 seconds (Rickard et al. 2000). Samples were allowed to sit 60 minutes to let coaggregates settle to the bottom of the tube. One hundred microliters of supernatant were removed from each sample and placed in a 96 well flat-bottom plate. Absorbance of the supernatant was measured at 620 (A) using a PerkinElmer 2030 workstation (Levin-Sparenberg et al. 2016). All coaggregation and autoaggregation assays were conducted in triplicate. *S. gordonii, S. oralis*, and *A. oris* pairs were used as positive controls on all 96-well plate assays.

Results of the high-throughput screen were used to calculate mean absorbance and associated 95% confidence intervals for each strain pair. Because strains were set to the same optical density (1.5) before combining, an expected value for the combined pair was calculated based on the average experimental OD of the two components. The mean absorbance, 95% confidence interval, minimum and maximum for each pair was then compared with the expected value for the pair. Coaggregation was suspected when the upper limit for the 95% confidence interval was below the expected value and was considered when the mean OD was below the expected value (Levin-Sparenberg et al. 2016).

Example Calculation of Expected Value:

CONTENTS	N	Mean over 3 trials	ODBUG 1	ODBUG 2	EXPECTED VALUE
CDC_F657+CDC_F657	3	0.31	0.31	0.31	
S. aureus 1263+S. aureus 1263	3	0.39	0.39	0.39	
CDC_F657+S. aureus 1263	3	0.23	0.31	0.39	(0.31+0.39)/2= 0.35

The empirically determined mean OD over the three replicates of CDC_F657+*S. aureus* 1263 was 0.23 (95% CI: 0.18, 0.28) which is less the expected value for the pair based on the average of the empirically determined OD for each of the components. As the value is less than the expected value this suggests that CDC_F657+S. aureus 1263 coaggregate.

3.3.3 FlowCam™ Imaging and Quantification of Coaggregation

Following an initial screen of our strain pairs using the high-throughput method, we identified pairs where the mean observed OD for the pair was less than the expected value. These pairs were further evaluated for coaggregation using FlowCam™ technology (Fluid Imaging Technologies, ME, USA), which uses flow cytometry to measure particle size over time (Levin-Sparenberg et al. 2016). *P. aeriginosa, H. influenzae 007.2.12 and 1247, S. aureus 1263, S. pneumoniae* serotype 10, and *N. meningitidis* were harvested from batch cultures and washed as described above. The washed pellets were suspended in coaggregation buffer to achieve an optical density of 1.0 (±0.1) at 600 nm. Prior to loading the cells into the FlowCam device, cell suspensions were further diluted 10X in coaggregation buffer to prevent clogging of the flow cell. We experimented with both 5X and 10X dilution and found a 5X dilution factor resulted in repeated clogging of the flow cell (Levin-Sparenberg et al. 2016; Segaloff et al. 2014). The first species was added to the device and was pumped through until it reached the flow cell. Data collection began once the Olympus UPlanFL N 10X/0.30 objective was successfully focused on the flowing particles. The second species was added to the vessel containing the first species and gently mixed one minute after initiation of data collection. FlowCam was run at a flow rate of 0.3 ml/min with images acquired at a rate of 10 frames per second, until the entire sample ran through the machine (45-55 min). Flash duration was set to 8 µSec. Particle size was measured using area based diameter (ABD) and a particle filter of 0.5 to 10,000 µM. Visual spreadsheet software was used for data collection.

3.3.4 Confocal Microscopy

As an additional visual test of autoaggregation and coaggregation between strains, 300 µl of each bacterial suspension in coaggregation buffer were stained with either

syto-9 (green: Excitation: 488, Emission: 503) or syto-61 (red: Excitation: 561, Emission: 645) nucleic acid stains (Invitrogen, Carlsbad, CA, USA). Each bacterial suspension was incubated for 30 minutes at room temperature to allow staining of the cells. Cells were washed three times with 1X PBS (phosphate buffer solution) and collected by centrifugation, as described above. Each bacterial strain was re-suspended in coaggregation buffer and combined for coaggregation. Autoaggregation studies consist of only one color stained cells. Twenty microliters of each sample were added to the slide and viewed under the microscope. The entire droplet was scanned and a minimum of three representative fields of view were captured for each pair and for each strain alone using Leica confocal laser scanning microscopy (CLSM, SPE, Leica, IL, USA) with a HCX PL APO 40X/0.85 CORR CS objective. Staining and microscopy were repeated twice to ensure consistency of results. Once the microscopy images were taken, the image files were rendered using Imaris (Bitplane, Zurich, Switzerland) computer imaging software.

3.4 Results and Discussion

3.4.1 Autoaggregation not detected by high-throughput screen

None of the *B. pertussis* or commensal strains showed autoaggregation on the high-throughput screen. However, when examined using confocal microscopy, autoaggregation was apparent in *N. meningitidis*. Autoaggregation in N. meningitidis is mediated by PilX, a type IV pilus-associated pilin-like protein (Lappann et al. 2006).

3.4.2 High-throughput screen identifies potentially coaggregating pairs

Using the high-throughput screening method, we examined 133 unique pairs of bacteria for coaggregation and autoaggregation. Each pair was screened at least three times, and as many as 33 times, with an average of six screens per pair. Based on an experimentally determined OD value that was significantly less than the calculated expected value for the pair, *H. influenzae* (strains 007.2.12, I247, I316, K29RE2.10), *P.*

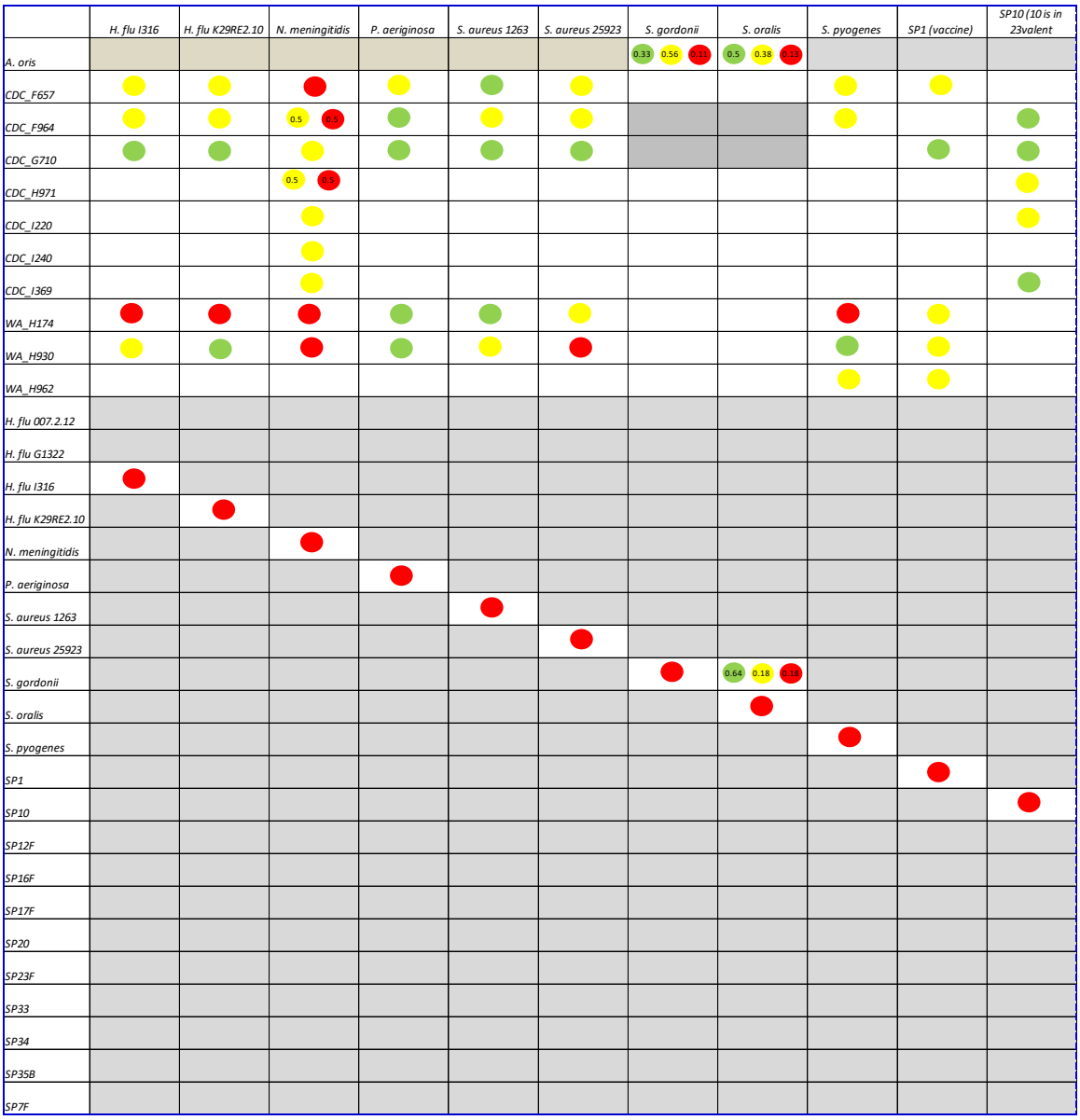
aeriginosa, S. aureus (strains 1263 and 25923), *S. pyogenes*, and *S. pneumoniae* (serotypes 1, 10, 16F, 20, 23F, 33, 34, 35B) were suspected as coaggregating with at least one of the ten *B. pertussis* strains tested (green circles, Figure 3.1). In addition *S. pneumoniae* 7F and 12F, *N. meningitidis*, H. *influenzae* G1322 also potentially coaggregated with at least one strain of pertussis based on OD values less than the expected values, although not significantly so (yellow circles, Figure 3.1).

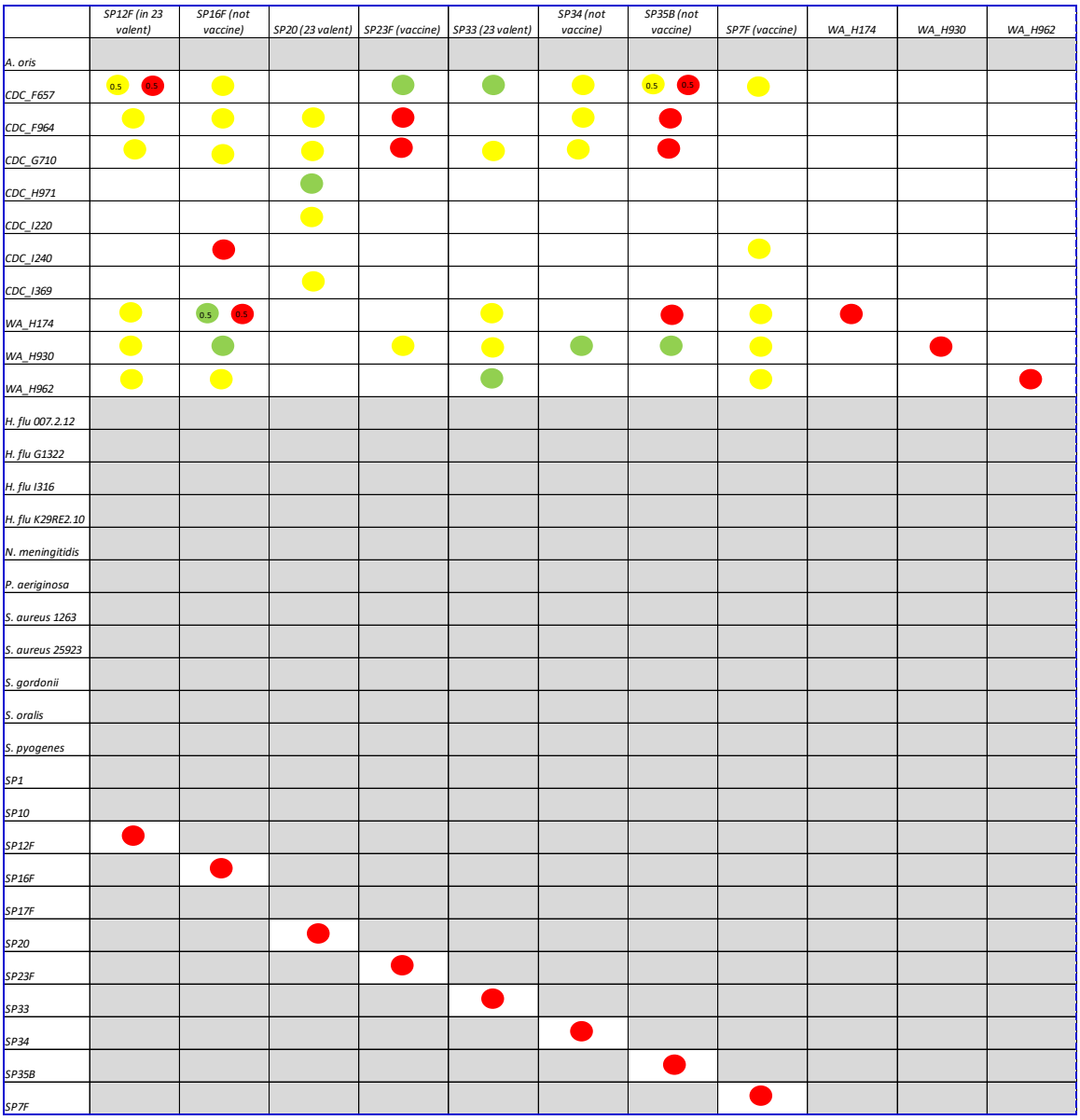
Results were quite variable. An organism would coaggregate with one strain of *B. pertussis*, but not others, or coaggregated with a particular strain one day and not the next. This variation is strikingly evident when examining the results of the control strain pairings, *A. oris+ S. oralis*, *S. oralis + S. gordonii*, and *A. oris + S. gordonii* (Figure 3.1). Although these strains are known to strongly coaggregate, results varied by day from coaggregating significantly to not at all. Many factors could contribute to between day variations: bacteria are harvested from separately grown batch cultures, which may differ slightly in length of growth time, exact nutrient content and pH of media, pH of buffers used for washing and re-suspension, number of times a strain has been passaged before current growth, and natural biological variation in amount of autoaggregation or coaggregation within a given species or species pair.

Based on the results of the high-throughput screen, summarized in Figure 3.1, and visual assessment of test crosses, we selected one *B. pertussis* strain, CDC-F567, and 7 test strains (*H. influenza* I247, *N. meningitidis*, *P. aeriginosa*, *S. aureus* 1263, and *S. pneumoniae* serotypes 10 and 16F, and *S. pyogenes*) for further analysis using FlowCam™ and confocal microscopy.

Table 3.1 Summary of high-throughput coaggregation screen results. Green circles represent OD values significantly less than expected value. Yellow dots represent OD values less than expected values. Red dots represent no difference between OD and expected value or OD higher than expected value.

	A. oris	*CDC_F657*	*CDC_F964*	*CDC_G710*	*CDC_H971*	*CDC_I220*	*CDC_I240*	*CDC_I369*	*H. flu 007.2.12*	*H. flu I247*	*H. flu G1322*
A. oris	●										
CDC_F657		●							●	●	●
CDC_F964			●						●		●
CDC_G710				●					●		●
CDC_H971					●						
CDC_I220						●					
CDC_I240							●				
CDC_I369								●			
WA_H174									●		●
WA_H930									●		●
WA_H962											
H. flu 007.2.12									●		
H. flu G1322											●
H. flu I316											
H. flu K29RE2.10											
N. meningitidis											
P. aeriginosa											
S. aureus 1263											
S. aureus 25923											
S. gordonii											
S. oralis											
S. pyogenes											
SP1											
SP10											
SP12F											
SP16F											
SP17F											
SP20											
SP23F											
SP33											
SP34											
SP35B											
SP7F											


H. flu I316
H. flu K29RE2.10
N. meningitidis
P. aeriginosa
S. aureus 1263
S. aureus 25923
S. gordonii
S. oralis
S. pyogenes
SP1 (vaccine)
SP10 (10 is in 23valent
A. oris
CDC_F657
CDC_F964
CDC_G710
CDC_H971
CDC_I220
CDC_I240
CDC_I369
WA_H174
WA_H930
WA_H962
H. flu 007.2.12
H. flu G1322
H. flu I316
H. flu K29RE2.10
N. meningitidis
P. aeriginosa
S. aureus 1263
S. aureus 25923
S. gordonii
S. oralis
S. pyogenes
SP1
SP10
SP12F
SP16F
SP17F
SP20
SP23F
SP33
SP34
SP35B
SP7F
0.33
0.56
0.11
0.5
0.38
0.13
0.5
0.5
0.64
0.18
0.18



3.4.3 FlowCam results inconclusive

The FlowCam™ device allows individual particles to be photographed and the particle characteristics measured. As a coaggregation assay it can be used to measure the change in particle size over time (Levin-Sparenberg et al. 2016). None of the *B. pertussis* pairings appeared to strongly coaggregate in the FlowCam™ device. The large

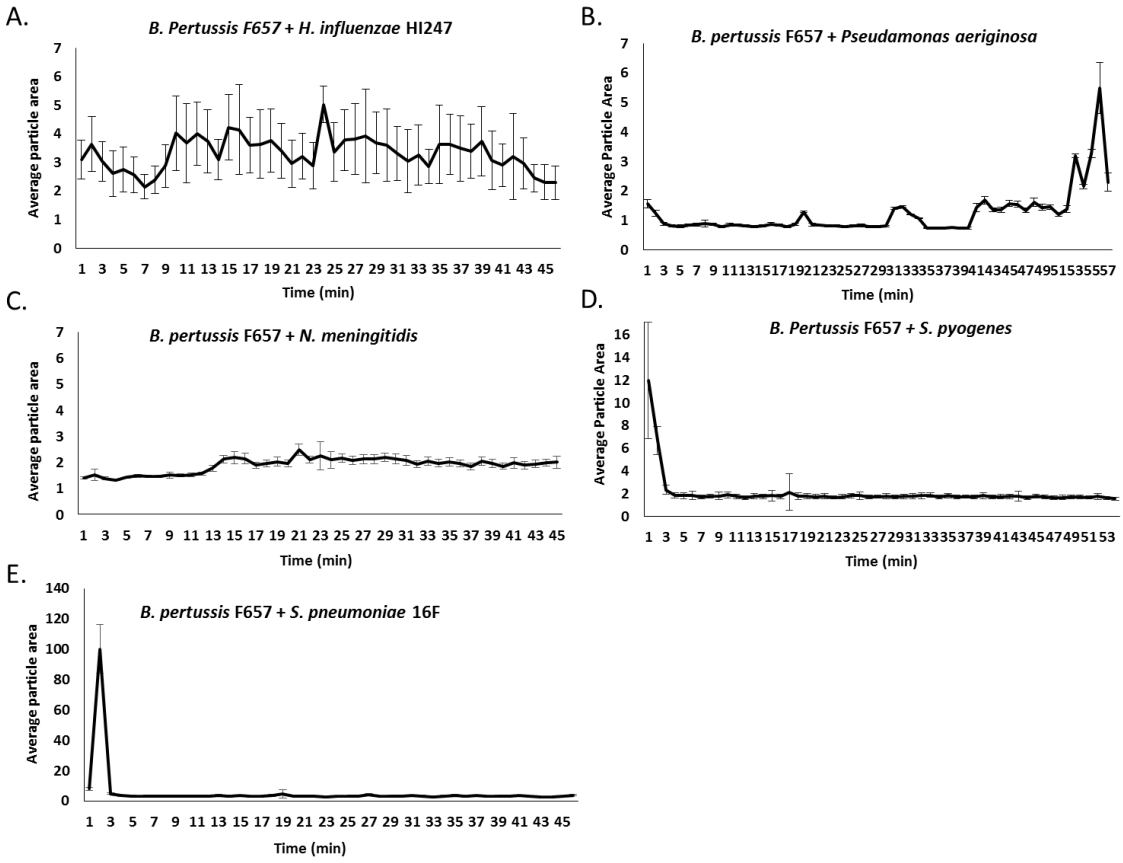
confidence intervals in the *B. pertussis* + *H. influenzae* H1247 pairing are reflective of the large variability of particle sizes through the flow cell at a given time point (Figure 3.2, Panel A). This indicates a combination of single bacterial cells and cell aggregates. Any cell aggregates formed are not large enough to rapidly drop out of the suspension resulting in no significant time component. Coaggregation in this pair was not strong enough to detect with a visual assay. Another study that examined coaggregation between *H. influenzae* and 30 other species also detected no visual coaggregation with *H. influenzae* (Stevens et al. 2015). In the *B. pertussis* F657+ *P. aeriginosa* pairing, there were significant increases in particle area starting around minute 53 (Figure 3.2, Panel B). In the combination of *B. pertussis* F657+ *N. meningitidis* a slight, although statistically significant, increase in particle area began at minute 14 (Figure 3.2, Panel C). In combinations of *B. pertussis* F657 with *S. pneumonia* 16F and S*. pyogenes*, a significant increase in particle size occurred immediately followed by a sharp decrease in particle size (Figure 3.2, Panel D and E). This could potentially occur if larger particles immediately fall out of suspension and are captured in the flow cell first, leaving only single cells remaining.

As with the visual assay and high-throughput screening method, there was a lot of run to run variation. Runs with stronger aggregation were more likely to be thrown out due to the larger particle sizes clogging the flow cell and interrupting the run. While the second bacterial species was added one minute after initiation of data collection to create a separation between *B. pertussis* alone and when combined with another species, increases in particle size could be due to either coaggregation or either species inducing autoaggregation in the other. Due to inconsistencies between runs and issues with clogged flow cells, we were unable to reach any general conclusions as to the ability of *B. pertussis* F657 to coaggregate with these other species.

In an attempt to clarify our conclusions and obtain more consistent results we tested the effect of changing growth times for the bacteria by reducing growth time from 24 hours to 18 hours for the test strains, and increased growth time from four to five days for *B. pertussis*. We found no noticeable effect on our results by manipulating

growth times. We also experimented with changing the dilution factor from 1:10 to 1:5 in hopes of inducing coaggregation. This resulted in blocked flow cells which may have been a result of increased coaggregation, but also resulted in unusable data. We also assessed the effects of switching from coaggregation buffer to PCR-grade water and from the use of Schaedler's broth to BHI broth for growth of test strains, but found no noticeable difference in results.

Figure 3.1 Average particle area per minute with associated 95% confidence intervals as measured with FlowCam device.



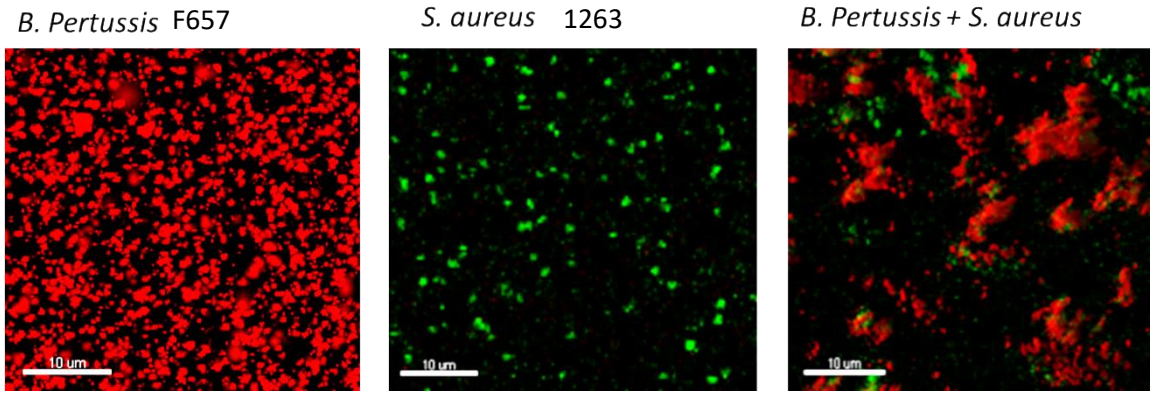
3.4.4 Confocal microscopy

To further understand the interactions between *B. pertussis* and other nasopharyngeal commensals, we stained our strains using Syto-9 (green) or Syto-61 (red) nucleic acid stains before crossing them for coaggregation, and then visualized using a confocal microscope. Each strain was also imaged on its own to rule out any autoaggregation. *N. meningitidis* appears to be the only strain that auto-aggregated (Figure 3.3, panel C). If coaggregation were present, we would expect to see large clusters of overlapping green and red cells, which was not apparent in any of our crosses. Mixing *B. pertussis* with *S. aureus* appeared to result in auto-aggregation of *S. aureus* (Figure 3.3, panel A). Mixing *B. pertussis* with *H. influenzae* HI247 appeared to result in slight auto-aggregation in *H. influenzae* (Figure 3.3, panel B). Crossing *B. pertussis* with *N. meningitidis* appeared to enhance autoaggregation of *N. meningitidis* (Figure 3.3, panel C). Combining *B. pertussis* with *P. aeriginosa* induced autoaggregation in both strains (Figure 3.3, panel D). The combination of *B. pertussis* with *S. pneumoniae* serotype 10 appeared to induce slight autoaggregation in *S. pneumoniae* (Figure 3.3, panel E.)

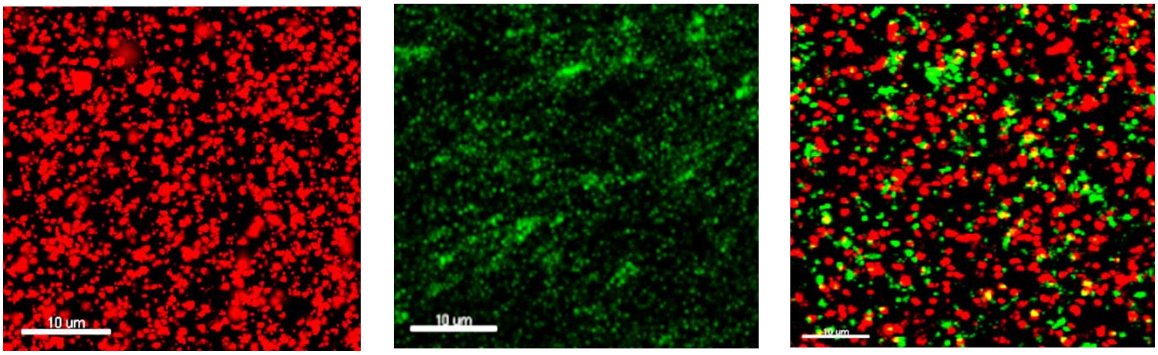
Based on our findings that the presence of *B. pertussis* may result in autoaggregation of one or both species, we attempted to determine if *B. pertussis* was excreting a molecule that induces autoaggregation. For this experiment we grew *B. pertussis* and suspended the cells in coaggregation buffer as described in the methods. The cells were allowed to remain in the buffer for an hour and then were filtered out. The buffer was saved and added to the other test strain cells in the FlowCam™ device rather than adding the *B. pertussis* cells themselves. This method did not induce auto-aggregation in the test strains.

Figure 3.2. FlowCam Visualization of selected bacterial pairings using confocal microscopy. Confocal microscopy images are represented in the x-y plane. Nucleic acid stains syto-9 (green) and syto-61 (red) was used to detect autoaggregation and coaggregation of bacterial strains. Bars represent 10 µM.

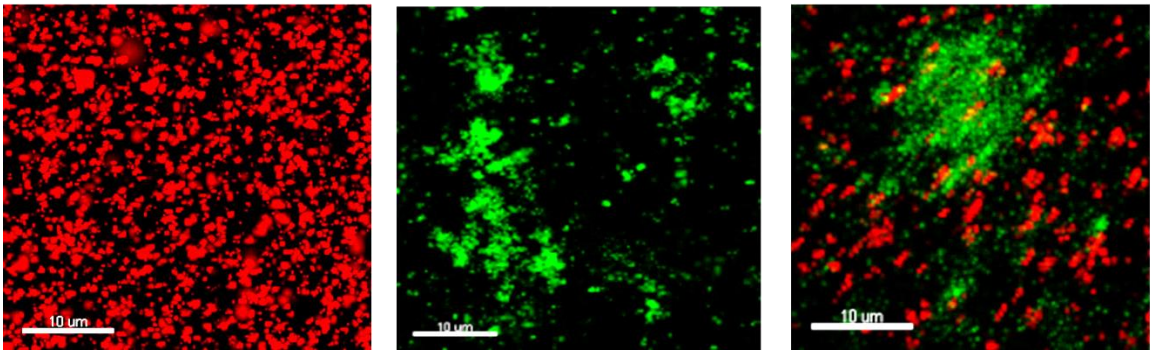
A.



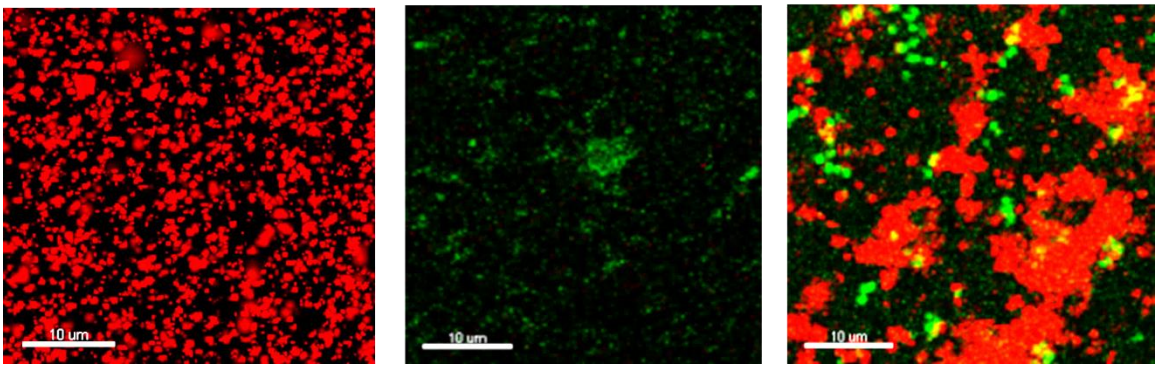
B. *B. pertussis* F657 *H. influenzae* HI247 *B. Pertussis + H. influenzae*



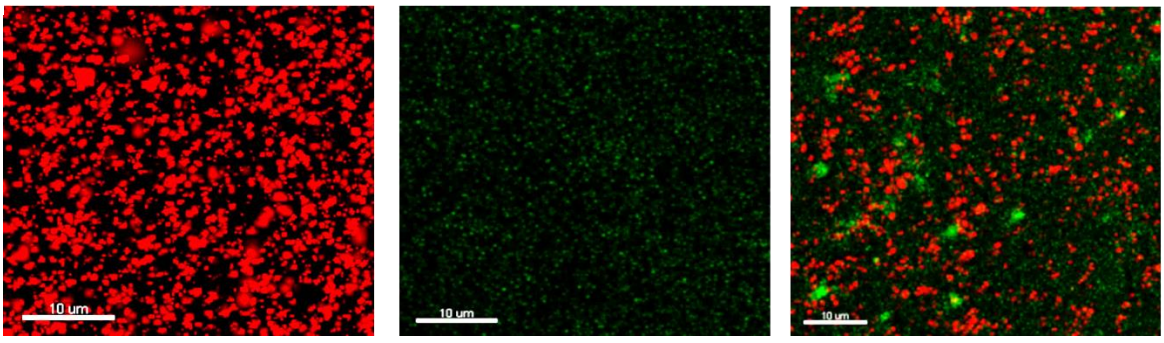
C. *B. pertussis* F657 *N. meningitidis* *B. Pertussis + N. meningitidis*



D. *B. pertussis* F657 *P. aeriginosa* *B. Pertussis + P. aeriginosa*



E. *B. pertussis* F657 *S. pneumoniae* 10 *B. Pertussis + S. pneumoniae*



3.4.5 Conclusion

We did not identify autoaggregation in any of the *B. pertussis* or commensals strains with the use of the high throughput screen. Further screening with confocal microscopy identified autoaggregation in *N. meningitidis*.

While there appears to be an interaction between *B. pertussis* and selected commensals, we conclude that it is not due to coaggregation between *B. pertussis* and the nasopharyngeal test strains. Based on our confocal microscopy results, the co-occurrence of *B. pertussis* with one of the test strains may induce auto-aggregation in one or both strains, although we were unable to confirm this.

Superinfection with other respiratory pathogens such as *H. influenzae*, *S. pneumoniae*, and *S. aureus* is common during and after infection with *B. pertussis,* which hints that polymicrobial interactions may be an important aspect of *B. pertussis* infection (Sawal et al. 2009). The specific mechanism of interaction between *B. pertussis* and the resident bacteria is unknown. *Bordetella* species contain many mechanisms for interbacterial competition that could be at play, including a type IV secretion system for secretion of pertussis toxin, bacteriophages, and potentially contact-dependent growth inhibition (Weyrich et al. 2014). Coaggregation is common among oral bacterial and would be a reasonable mechanism if certain resident microorganisms are necessary for *B. pertussis* to attach. However if the findings from the murine model hold in humans, that the presence of nasopharyngeal microbiota prevents efficient colonization by *B. pertussis*, antagonistic coaggregation interactions could be responsible, or as we saw some evidence of in our results, induced autoaggregation on one or both species in the presence of *B. pertussis*.

To the authors' knowledge, this is the first study of coaggregation in *B. pertussis*. While we did not find evidence to support coaggregation between *B. pertussis* and the organisms studied, we did find some indication that autoaggregation of one or both species is induced in the presence of *B. pertussis*. Further study is needed to better understand the role of microbiota in aiding or abating *B. pertussis* infection in humans.

A better understanding of how the microbiota is involved could result in better treatment and prevention methods for *B. pertussis* infection.

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Chapter 4. Epidemiologic Evidence for Co-colonization of Selected Commensals and Asymptomatic *B. pertussis* Carriage

4.1 Abstract

We hypothesized that the presence of commensals in the oropharynx might enhance or decrease *Bordetella pertussis* colonization. To test this hypothesis, we planned to screen an archival collection of nasopharyngeal swab specimens collected as part of a cross-sectional study of nasopharyngeal *S. pneumonia* carriage in Phu Vinh hamlet of Vinh Thanh commune, Nha Trang district, Vietnam during October, 2006, for asymptomatic *B. pertussis* carriage and that of selected commensals. Since the Expanded Programme on Immunization was introduced in Vietnam in 1981, vaccination rates with the whole cell vaccine increased and remain high. Four samples were positive for *B. pertussis,* but the prevalence of *S. aureus* was significantly less than expected based on previous studies suggesting that during handling or storage that the quality of the samples was compromised. Thus it is likely that the estimated prevalence of *B. pertussis* is also in error. Due to issues with sample quality, the study ended early with inconclusive results.

4.2 Introduction

The United States has seen a dramatic increase in the number of reported *B. pertussis* cases since the introduction of the acellular vaccine in the 1990s (Centers for Disease Control and Prevention 2016). Some studies have proposed asymptomatic transmission from individuals vaccinated with acellular vaccines as a potential mechanism for the increase in number of cases although little is known about the actual

prevalence of asymptomatic carriage (Althouse & Scarpino 2015; Zhang et al. 2014; Cortese et al. 2007; de Melker et al. 2006). Another important aspect of this resurgence is that a large proportion of these cases have occurred in fully vaccinated individuals making it clear that factors outside of vaccination status also influence who becomes infected (Centers for Disease Control and Prevention 2016). We hypothesized that bacterial commensals in the nasopharynx of potential hosts might enhance or decrease the risk of *B. pertussis* colonization. We screen archival nasopharyngeal swabs collected from healthy individuals to estimate the prevalence of asymptomatic carriage of *B. pertussis* and co-occurrence with select commensals.

4.2.1 Asymptomatic Carriage as a Mechanism for Resurgence in Reported Cases

Warfel et al. propose that asymptomatic infection and subsequent transmission by individuals vaccinated with the acellular vaccine could, in part, explain the increase in observed *B. pertussis* incidence (Warfel et al. 2014). Using a baboon model for *B. pertussis* infection, Warfel et al. and found that while vaccination with acellular pertussis (ap) protected against severe symptoms, it did not protect against colonization (Warfel et al. 2014). Moreover, vaccinated animals did not clear the infection any faster than naïve animals and could readily transmit *B. pertussis* to their unvaccinated contacts (Warfel et al. 2014). By comparison, more rapid clearance was induced by the whole cell vaccine and animals that were previously infected were not colonized upon secondary infection (Warfel et al. 2014). In addition, re-analyses of aP efficacy studies using more sensitive serologic diagnostic criteria found that while pertussis toxoid alone protects against typical laboratory-confirmed pertussis infection, it does not protect against colonization or infection (Storsaeter et al. 1990). To provide further evidence for the importance of asymptomatic transmission, Althouse and Scarpino used wavelet analysis of *B. pertussis* incidence and phylodynamic analysis of clinical isolates. Their analysis of the sequences showed more genetic diversity in the bacterial population than could be explained by the observed number of infections without the possibility of

asymptomatic carriage and that the time of changes in age-specific attach rates were consistent with asymptomatic transmission (Althouse & Scarpino 2015).

While these studies point out the potential importance of asymptomatic carriage in transmission dynamics, only a limited number of studies aimed at estimating the prevalence of asymptomatic carriage have been conducted. Using IgG and IgA antibodies to pertussis toxin (PT) and filamentous hemagglutinin (FHA), von Linstow et al. found that 5-10% of a cohort of vaccinated, healthy children had evidence of *B. pertussis* infection during their first year of life despite few clinical cases recognized in the community at the time (von Linstow et al. 2010). In a sample of nasopharyngeal swabs from asymptomatic Chinese school children ages 7 to 15, 2/629 (0.3%) were both culture-positive and PCR-positive and 30/269 (4.8%) were PCR-positive (Zhang et al. 2014). Although the prevalence was low, positive samples were found in all four counties studied (Zhang et al. 2014).

To understand if the prevalence of asymptomatic carriage has increased with the introduction of acellular vaccines, it is important to have estimates of the prevalence in countries where vaccination rates with the whole cell vaccine are high as a comparison. The Expanded Programme on Immunization (EPI) was introduced in Vietnam in 1981 (Jit et al. 2015). By 2009, 96% of children under age one had received three or more doses of whole cell pertussis vaccine (Jit et al. 2015; Anh et al. 2015). We screened archival nasopharyngeal swabs collected from healthy individuals in Vietnam to estimate the prevalence of asymptomatic carriage in this highly vaccinated population.

4.2.2 Nasopharyngeal Commensals as a Determinant of *B. pertussis* Infection

Superinfection with other respiratory commensals such as *H. influenza*, *S. pneumoniae*, and *S. aureus* is common during and after infection with *B. pertussis* hinting that polymicrobial interactions may be an important aspect of *B. pertussis* infection (Sawal et al. 2009). As further evidence of the importance of nasopharyngeal commensals as a determinant of pertussis infection, Weyrich et al. demonstrated that

treating mice with antibiotics to remove resident microorganisms from the murine nasal cavity greatly enhanced *B. pertussis* colonization. For *B. pertussis* to colonize in presence of even a single naval cavity bacterial species required ten times the dose needed in antibiotic treated mice (Weyrich et al. 2014). This led us to hypothesize that specific interactions between *B. pertussis* and nasopharyngeal commensals might enhance or decrease *B. pertussis* colonization and help explain why vaccinated individuals might still asymptomatically carry *B. pertussis.*

In a preliminary screen for coaggregation (specific recognition and adherence between distinct bacterial species) between 10 different *B. pertussis* strains with common nasopharyngeal commensals: *S. pneumoniae, S. aureus, H. influenzae, S. pyogenes, P. aeriginosa,* and *N. meningitidis*, we obtained results consistent with coaggregation between *B. pertussis* and *H. influenzae*, *P. aeriginosa, S. aureus*, *S. pyogenes*, and *S. pneumoniae* with the use of our high-throughput, quantitative screening method (Levin-Sparenberg 2016). Based on further analysis using FlowCam™ and confocal microscopy, we did not find evidence to support coaggregation between *B. pertussis* and the organisms studied, but did find some indication that autoaggregation of one or both species is induced in the presence of *B. pertussis* (Levin-Sparenberg 2016). Autoaggregation was particularly apparent between *B. pertussis* and *P. aeriginosa* and *S. aureus* species (Levin-Sparenberg 2016). The purpose of this study was to determine if there was evidence that these *in vitro* interactions between *B. pertussis* and selected commensals also occurred *in vivo*.

4.2.3 Study Objectives

This study had two objectives: 1) to assess the prevalence of asymptomatic *B. pertussis* carriage in a population with high whole cell vaccination rates, and 2) to determine if specific nasopharyngeal commensals co-occur with *B. pertussis* in the nasopharynx of healthy individuals in an effort to better understand why some individuals get colonized while others do not. For this study, we screened archival

nasopharyngeal swabs from a sample of 519 healthy individuals collected from Phu Vinh hamlet of Vinh Thanh commune, Nha Trang district, Vietnam during October, 2006, to estimate the prevalence of asymptomatic carriage of *B. pertussis* in a population with high whole cell vaccination rates. We additionally screened for the prevalence of *H. influenzae, S. pneumoniae, P. aeriginosa,* and *S. aureus* to determine if they co-occur with *B. pertussis.*

4.3 Methods

4.3.1 Archival Collection

The archival nasopharyngeal swabs were collected as part of a cross-sectional study of nasopharyngeal (NP*) S. pneumonia* carriage in Phu Vinh hamlet of Vinh Thanh commune, Nha Trang district, Vietnam during October, 2006 (Talerico et al. 2009). Households in Phu Vinh with at least one child five years or younger and households with adults only (≥ 18 years) were randomly selected from a detailed census list compiled by the Khanh Hoa Provincial Health Service and invited to participate. Households were recruited by trained interviewers until 75 households with children (≤ 5 years) and 40 households comprised of only adults were enrolled. All current household members were invited to participate in an interview survey and NP swab procedure. Out of the 146 households that were approached, 115 participated (79%). Out of the 96 households with young children that were approached, 75 (78%) agreed to participate. Forty households containing only adults, out of the fifty households approached, agreed to participate (80%). The reason most commonly cited for refusal was that all household members were not available to participate due to extensive travel required for employment (Talerico et al. 2009).

NP swab procedures took place at the Vinh Thanh Commune Health Center and were obtained from all participants by trained physicians, in accordance with World Health Organization recommendations (O'Brien et al. 2003). Three households, which previously contained only adults, switched to the households with one child ≤ 5 years group in the analysis due to the birth of a child between recruitment and survey

administration. Therefore, 78 households contained at least one child ≤ 5 years and 37 households contained only adults (≥ 18 years) (Talerico et al. 2009).

Specimens were collected using calcium alginate-tipped wire swabs (Calgiswab® Type 1, Puritan Medical Products Company LLC, Guilford, ME). Swabs were passed through the anterior nares to the back of the nasopharynx, held in place for two seconds, and rotated 180° before removal. After collection, swab specimens were placed in STGG transport media and held at 4°C for no more than four hours at the Commune Health Center before being transported to the local hospital where they were stored at -20°C for one week (Talerico et al. 2009). NP swabs were ultimately shipped on dry ice to the University of Michigan and stored at -80°C (O'Brien et al. 2003).

Informed consent for the original study was obtained from all adults (≥ 18 years) and parents of children prior to participation (Talerico et al. 2009). In addition, oral assent was obtained from participants 6-17 years old prior to the NP swab procedure (Talerico et al. 2009). All study procedures were approved by the Health Sciences Institutional Review Board (IRB) at the University of Michigan (HUM00006257), the IRB of the National Institute of Hygiene and Epidemiology in Hanoi, Vietnam, and the IRB of the International Vaccine Institute in Seoul, Korea (2006-009). Use of the archival swab samples for this project received a determination of "Not Regulated" Status by the University of Michigan (HUM00115197).

4.3.2 Laboratory Methods

DNA was extracted using a QIAGEN (Venlo, Netherlands) DNeasy Blood & Tissue kit. One hundred microliters of each sample was first incubated at 37°C with an 80 µL enzyme cocktail comprised of Promega cell lysis solution (Madison, WI, USA), lysozyme, mutanolysin, RNase A, and lysostaphin (Sigma Aldrich) in 22.5:4.5:1.125: 1.125:1 parts, respectively for 30 minutes. After extraction, DNA quantity and quality was measured using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at −80°C.

4.3.3 Identification of *B. pertussis* and commensals

To identify *B. pertussis*, we first screened for *IS481*, an insertion sequence found in multiple copies in *B. pertussis*, and then screened positive samples with primers specific to pertussis toxin subunit S1 (*ptxS1*), *B. parapertussis* IS*1001* (pIS*1001*), and *B. holmesii* IS*1001*-like (hIS*1001*) targets (Table 1) (Tatti et al. 2011). This combination of primers can be used to distinguish *B. pertussis* from other Bordetella species (Table 2). Samples positive for *B. pertussis* were also screened for *P. aeriginosa* using primers for *gyrB* and *S. aureus* with primers specific to *nucA* (Table 1) (O G Brakstad 1992; Lee et al. 2011). A random sample of the remaining isolates (n=213) was screened for *P. aeriginosa* and *S. aureus* to determine the background rate of these organisms in the collection. We used a 50 µl reaction mixture for all reactions composed of 25 µl GoTaq Green Master Mix 2X, 1 µl forward primer, 1 µl reverse primer, 15 µl sample DNA, and 8 µl PCR-Grade H_20. The following program was used to amplify *IS481*: 1 = 95°C for 2 min, 2 = 95°C for 30 sec, 3 = 65°C for 30 sec, 4 = 72°C for 20 sex, 5 = GOTO 2 29 times, 6 = 4°C forever. Step 3 was switched to 57°C for ptxS1 and to 60°C for all other primer sets. PCR products were visualized on 2% agarose gels. All PCR reactions were conducted in duplicate.

To identify samples positive for *H. influenzae*, NP swabs from all participants were plated on chocolate bacitracin agar and incubated overnight at 37°C with 5% CO^2. Thirty colonies were picked and patched on chocolate agar plates without bacitracin. A porphyrin test was performed on each patch. Positive results were considered para-influenza. Samples were also plated on Brain Heart Infusion (BHI) agar with hemin only (X), NADH only (V) and hemin and NADH (X and V). Samples positive for *H. influenzae* will only grow on BHI with hemin and NADH. Stocks were prepared from the BHI (X and V) plates. PCR was performed on these samples using primers specific for lgA and LgtC. Samples were considered positive for *H. influenzae* if they were porphyrin negative, BHI (X and V) positive and IgA and LgtC positive (Patel 2009).

To identify samples positive for *S. pneumoniae*, NP samples from all participants were inoculated on trypticase soy agar with 5% sheep blood (TSA II) containing gentamicin (2.5 mg/L) and separately on non-selective media (TSA II). Plates were

incubated at 37°C in 5% CO^2 overnight (O/N). Presumptive identification of *S. pneumoniae* was based on colony morphology and α-hemolysis on blood agar (O'Brien et al. 2003). Confirmatory identification was based on optochin sensitivity (zone ≥14 mm with 6mm P discs after O/N incubation at 37°C in 5% CO^2), solubility in 10% deoxycholate using the plate method, and Gram stain ((O'Brien et al. 2003; Talerico et al. 2009). Capsular typing was performed as described elsewhere and also served as a confirmatory measure for presence of *S. pneumoniae* (Talerico et al. 2009).

Table 4.1. Sequences and amplicon lengths of primers used in PCR assays

Primer	Sequence (5' -> 3')	Amplicon length (bp)	Source
IS481	GAT TCA ATA GGT TGT ATG CAT GGT T	390	(Fry et al. 2009)
	TTC AGG CAC ACA AAC TTG ATG GGC G		
ptxS1	CGCCAGCTCGTACTTC	55	(Tatti et al. 2011)
	GATACGGCCGGCATT		
pis1001	TCGAACGCGTGGAATGG	65	(Tatti et al. 2011)
	GGCCGTTGGCTTCAAATAGA		
his1001	GGCGACAGCGAGACAGAATC	67	(Tatti et al. 2011)
	GCCGCCTTGGCTCACTT		
gyrB	GGCGTGGGTGTGGAAGTC	190	(Lee et al. 2011)
	TGGTGGCGATCTTGAACTTCTT		
nucA	GCGATTGATGGTGATACGGTI	270	(O G Brakstad 1992)
	AGCCAAGCCTTGACGAACTAAAGC		

Table 4.2. Algorithm for single target PCR assays

	IS481	*ptxS1*	*pIS1001*	*hIS1001*
B. pertussis	+	+	-	-
B. parapertussis [a]		+	+	
B. holmseii	+			+
B. pertussis + B. parapertussis [a]	+	+	+	
B. pertussis + B. holmseii	+	+		+

[a] A specimen positive for pIS1001 most likely contains *B. parapertussis*, but *B. bronchiseptica* cannot be ruled out. (Tatti et al. 2011; European Centre for Disease Prevention and Control 2012)

4.4 Results

Out of the 519 samples, four were positive for *IS481* (ID numbers 143014008, 143019605, 143019603, 143019604). The same four samples were also positive for *ptxS1* and negative for pIS1001 and hIS1001 and as such were identified as positive for *Bordetella pertussis*. These four samples were negative for *P. aeriginosa* and *S. aureus* based on PCR results and were negative for *S. pneumoniae* and *H. influenzae* based on historical data collected from the samples using the methods described above (Talerico et al. 2009).

Fifty-six of the 519 samples (11%) were positive for *S. pneumoniae* (Talerico et al. 2009). Thirty-nine samples were positive for H. influenzae (8%) (Patel 2009). *H. influenzae* was found only in samples that also contained *S. pneumoniae*.

To determine the number of samples we needed to screen to estimate the background carriage rates for *S. aureus* and *P. aeriginosa*, we used estimates of the *S. aureaus* carriage rate, which required the larger sample size, from the literature in a power calculation. In a meta-analysis conducted by Kluytmans et al. consisting of 18 cross-sectional studies of oropharyngeal *S. aureus* carriage in the general population representing 13,873 people, the mean carriage rate was estimated at 37.2% and ranged

from 19.0% to 55.1% (Kluytmans et al. 1997). In a study of 497 healthy children ages 6-17, 53.1% were identified as *S. aureus* carriers (Esposito et al. 2014). Based on our power calculation, we needed to screen 200 samples. Of the first 88 samples screened, none were positive for *S. aureus*. As this was highly-inconsistent with the literature, no further samples were screened.

4.5 Discussion

This study aimed to determine the prevalence of *B. pertussis* carriage in a sample of healthy Vietnam residents and to determine its co-occurrence with other common nasopharyngeal commensals. As we only found four samples that were positive for *B. pertussis*, less than we expected based on the sparse literature available, we were severely underpowered to make any statistically valid estimates of co-occurrence between *B. pertussis* and any of the commensals. Ideally, in addressing this question, one would sample based on *B. pertussis* case status to have more power to detect commensals correlated with infection status. However, we were also interested in determining the prevalence of asymptomatic *B. pertussis* carriage so this sample addressed our needs for that question. Consistent with the literature, we did find that none of the commensals we screened for (*H. influenzae, S. pneumoniae, S. aureus, P. aeriginosa*) were present in the samples that were positive for *B. pertussis* (Weyrich et al. 2014).

Expected background rates for the commensals were taken from the literature to estimate the number of samples in our study that we would expect to see positive for each of the species. This was used to calculate the number of specimens from our sample that should be screened to calculate background rates for each of the samples in our collection. Based on the literature, we expected roughly 40% of our specimens to be positive for *S. aureus* (Kluytmans et al. 1997; Ebruke et al. 2016; Sivaraman et al. 2009; Esposito et al. 2014). After screening the first 88 specimens of the 213 specimen sample we generated to calculate background rates for each of the commensals, none were found positive for *S. aureus*. This is highly inconsistent with the literature. As such

the historical specimens used were deemed unreliable for additional study, and the study ended.

It is likely that the true number of positive *B. pertussis* samples in the collection is higher than we were able to detect. The DNA was tested for viability at the beginning of the experiment by taking a random sample of specimens deemed positive for *S. pneumoniae* by the researcher who originally collected the samples, and then screening for *S. pneumoniae* using PCR for the *lytA* gene. Out of the ten presumptive positive samples that we screened, nine resulted in positive determination giving us the false impression that the rest of the samples would be viable. Questionable quality is an inherent risk of archival samples, and further screening of the samples before use in the study could have prevented this outcome.

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Chapter 5. Effects of Widespread Vaccination on the *Bordetella pertussis* Genome

5.1 Abstract

Following the implementation of widespread vaccination in the 1940s in the United States, the number of reported pertussis cases in North America decreased by over 90%; however, since introduction of the acellular vaccine in the 1990s, the annual number of reported pertussis cases in the United States has increased 18-fold. The reason for this increase is not entirely clear; one hypothesized mechanism is antigenic divergence of circulating strains in response to selective pressures from vaccines. We conducted whole genome sequencing on a collection of 100 isolates dating from 1935 (pre-vaccine era) to 2013 (current vaccine components and schedule). Isolates were randomly selected from 8 different vaccine-related time periods: 1935-1945 Pre-vaccine era, 1946-1969 Early whole cell vaccine era, 1970-1990 Late whole cell vaccine era, 1991-1996 Combination of whole cell vaccine and acellular vaccine for 4th and 5th doses, 1997-1999 Early acellular vaccine era, 2000-2002 Middle acellular vaccine era, 2003-2005 Late acellular vaccine era, and 2006-2013 Tdap booster era. Across all samples, this collection varied from the Tohama 1 reference genome by 3,018 single nucleotide polymorphisms (SNPs). The phylogeny revealed 2 main clades and a distinct separation between the pre-vaccine era isolates and the rest of the collection ($p<0.0001$). These results support a significant effect of vaccination on extinction of strains resulting in a replacement of populations.

5.2 Introduction

Pertussis (Whooping Cough) is a highly infectious upper respiratory infection caused by the bacteria *Bordetella pertussis,* characterized by violent and uncontrollable

coughing (Halperin SA 2012). Although individuals of all ages can be infected, complications in infants are more common and can result in permanent disability or death (Halperin SA 2012). Widespread vaccination has decreased the number of reported pertussis cases in North America by over 90% since vaccination began in the 1940s (Halperin SA 2012); however, the past few years have seen a resurgence of pertussis cases in the United States that coincides with the introduction of the acellular vaccine (Centers for Disease Control and Prevention 2016). In 2012, 41,880 pertussis cases were reported, which is the highest number of reported cases in the United States since 1955 (Centers for Disease Control and Prevention 2016). Although a resurgence of pertussis has been reported in other highly vaccinated countries including Norway, Australia, and the Netherlands, there is considerable geographic heterogeneity in resurgence (Jackson & Rohani 2013). Spain and some other highly vaccinated countries have seen a significant decrease in cases, and Argentina has seen no significant change in pertussis incidence (Jackson & Rohani 2013).

The reason for the increase in number of pertussis cases reported in the US is not entirely clear. Some of the current hypotheses include: waning immunity over time following vaccination or infection (Tan et al. 2005; Wearing & Rohani 2009), decreased vaccination rates (Omer et al. 2009), variable vaccine efficacy (Crowcroft & Pebody 2006; Ntezayabo et al. 2003), age-structured contact patterns (Rohani et al. 2010), acellular vaccines (first implemented in 1991) protect against disease but not infection and thus fail to prevent transmission (Warfel & Merkel 2012), and antigenic divergence of circulating strains in response to selective pressures from vaccines (Mooi et al. 2001; Mooi et al. 2009; Marieke J. Bart et al. 2014; Bart et al. 2010). Evidence for these hypotheses has been reviewed elsewhere (Sealey et al. 2016; Burns et al. 2014).

This study will address the extent to which the current resurgence in reported cases in the United States can be explained by genetic variation in circulating strains in response to selective pressure from vaccines.

5.2.1 Vaccine Composition

The whole cell pertussis vaccine, first introduced in the United States in the 1940's was generally composed of 1-3 different strains, which varied between manufacturers and were selected from the predominant circulating types (Guiso 2009; Schmidtke et al. 2012). It is unclear specifically which strains were used. Due to concerns over reactogenicity of the whole cell vaccine, an acellular vaccine was introduced in the United States in 1991 as a safer alternative (Centers for Disease Control and Prevention 2016).

The current acellular pertussis vaccines contain five *B*. *pertussis* components: pertussis toxin (PT), filamentous hemagglutinin (FHA), fimbrial agglutinogens 2 and 3 (FIM2 and FIM3), and pertactin (PRN) (Decker & Edwards 2000). The two main manufacturers of the acellular vaccine used in the United States are GlaxoSmithKline and Sanofi Pasteur. The strain used for the manufacture of the GlaxoSmithKline vaccine in the United States is Tohama 1, a strain first isolated in Japan, which has an allelic variant profile of *prn (1)-ptxP(1)-ptxS1B-fim2(1)-fim3(A)* (Schmidtke et al. 2012; Bottero et al. 2007). The Sanofi Pasteur acellular pertussis vaccine is manufactured from *B. pertussis* strain 10536 (*prn(1)-ptxP(1)-ptxS1D-fim2(1)-fim3(A))*(Bottero et al. 2012)obtained from the Michigan Department of Health (Grabenstein 2012). Studies worldwide document antigenic divergence between vaccine strains and currently circulating isolates with respect to several of these surface proteins (Schmidtke et al. 2012; Cassiday et al. 2000; Kallonen & He 2009; Octavia et al. 2012; Advani et al. 2011; Bart et al. 2010; Borisova et al. 2007; Mooi et al. 1998). The main components of these acellular vaccines and the current predominately circulating allele in the United States are listed below (Table 5.1).

Table 5.1 Genotype of vaccine strains, number of circulating alleles, and predominant allele in United States.

	prn	*ptxP*	*ptxS1*	*fim2*	*fim3*	Sources
Tohama 1 genotype	1	1	B	1	A	Schmidtke et al. 2012 Bottero et al. 2012
10536 genotype	1	1	D	1	A	Bottero et al. 2012 Grabenstein 2012
Number of circulating alleles	2	11	6	2	4	Kallonen and He 2009 Mooi et al. 2009 Locht 2007
Predominate allele in US	2	3	A		B	Schmidtke et al. 2012 Cassiday 2000

5.2.2 Pertussis Particularly Susceptible to Vaccine-Induced Selection

Diseases like pertussis can be particularly susceptible to vaccine-induced selection because the acellular vaccine may not be completely effective at blocking transmission and there is an abundance of evidence that immunity wanes within a few years of vaccination creating leaky hosts (Warfel et al. 2014; Klein et al. 2012; Tartof et al. 2013; Mooi 2010; Read & Mackinnon 2007). When a vaccine fails to induce sterilizing immunity, natural selection has the opportunity to act on wildtype pathogens as they pass through vaccinated hosts allowing identification of weakness in vaccine-induced immunity (Read & Mackinnon 2007). In addition, the acellular vaccines only target 3-5 pathogen antigens (Read & Mackinnon 2007). By only targeting a subset of strains within a population, variants not included in the vaccine are given a competitive advantage (Read & Mackinnon 2007). Even without strain-specific effects, wide-spread vaccination reduces the number of hosts who are fully susceptible within a population thereby reducing the probability that *B. pertussis* will encounter and be amplified in a susceptible host. This dependency on the immune status of the host in determination of relative pathogen fitness also prompts pathogen evolution through variable selection (Read & Mackinnon 2007). While the mutations occur at random, selective pressures

can increase the frequency of advantageous mutations. When mutations arise in the surface antigens to which the vaccine has induced protective immunity against, the encoded protein structure may differ so greatly that it is not recognizable by the host immune system creating a vaccine-escape mutant (Read & Mackinnon 2007). Even under strong selection, advantageous mutations can go undetected within a population for decades before the balance is tipped in favor of vaccine-escape mutants (Read & Mackinnon 2007). The presence of vaccine-escape mutants fits with the epidemiologic profile of *B. pertussis*; in 2015 86% of pertussis cases under age 7 with known vaccination status had received at least one dose of vaccine (Centers for Disease Control and Prevention 2016).

5.2.3 Evidence for vaccine-driven evolution

Pertussis toxin (*ptxP1* allele) is a component of all acellular vaccines. The *ptxP3* allele was first identified in the United States in 1989 and since 1991 has been the dominant *ptxP* allele in the United States replacing the *ptxP1* allele found in both of the vaccine strains (Schmidtke et al. 2012). The *ptxP3* allele is associated with increased toxin production (Schmidtke et al. 2012; Mooi et al. 2009). Bart et al. examined the sequences of 343 *B. pertussis* strains that were collected from around the world during 1920-2010 and found that introduction of the acellular vaccine coincided with an expansion of the *ptxP3* lineage resulting from the acquisition of the *fim3–2* allele and with the decline of the *ptxA1* carrying lineage (Marieke J. Bart et al. 2014). These results support a vaccine-induced bottleneck resulting in a reduction in diversity followed by an expansion of the *ptxP3* genotype (Marieke J. Bart et al. 2014).

Pertactin, another component of acellular vaccines, is a surface adhesin involved in attachment of the bacterium to integrin proteins on the surface of ciliated epithelial cells in the nasopharynx (Halperin SA 2012). A transition from the vaccine allele of *prn1* to *prn2* as the dominate allele in the United States occurred during the early 1990's (Schmidtke et al. 2012; Cassiday et al. 2000). Recently there has been a dramatic

increase in the number of strains that do not express pertactin (prn), with large variation occurring between countries (Belcher & Preston 2015). The increase in prn-deficient strains seems to occur mainly in countries using the acellular vaccine. A study in Finland identified 2/76 strains as prn-deficient (Barkoff et al. 2012). In Japan 27% of 121 strains (Otsuka et al. 2012), in Australia 30% of 320 strains (Lam et al. 2014), and in the United States approximately 50% of the strains were identified as prn-deficient (Pawloski et al. 2014). In the global collection analyzed by Bart et al. (discussed above), no strains were found to be prn-deficient (Marieke J. Bart et al. 2014). The majority of these strains were isolated before 2008, which may indicate that prn-deficiency has arisen more recently (Marieke J. Bart et al. 2014). Those infected with Prn-deficient strains and those infected with prn-expressing strains have displayed no difference in clinical presentation (Martin et al. 2015). Interestingly, those infected with a prn-deficient strain were more likely to be fully-vaccinated suggesting there may be a selective advantage for this state in areas where acellular vaccination coverage is high (Martin et al. 2015). In a study examining *B. pertussis* clearance in mice, prn-expressing strains were cleared more rapidly than prn-deficient strains which suggests a potential fitness advantage to prn-deficiency in hosts vaccinated with the acellular vaccine (Hegerle et al. 2014).

In a study of 100 United Kingdom *B. pertussis* isolates dating from 1920-2012, with particular emphasis on isolates from the 2012 outbreak, Sealey et al. provided evidence that the genes encoding the acellular vaccine antigens are evolving more rapidly than genes encoding other cell surface proteins, based on a significantly higher frequency of synonymous and non-synonymous SNPs in vaccine antigen-encoding genes compared with cell surface protein-encoding genes, in all of the vaccine eras including the pre-vaccine era (Sealey et al. 2015). Even in the absence of vaccination, the immune response to these antigens likely creates selective pressure (Sealey et al. 2015). However, since the introduction of acellular vaccines, the acellular vaccine antigen-encoding gene evolution rates have significantly increased, which suggests that selective pressure on these antigens has increased since introduction of acellular vaccines (Sealey

et al. 2015). Bart et al. calculated SNP densities for different categories of genes and found that SNP densities were higher than the genome average in the "virulence" and "transport and binding" genes categories. Being on the surface of the bacteria, these genes are more likely to be subjected to selection pressure by the immune system.

The findings of these studies support the hypothesis of vaccine-induced divergent selection pressure on *B. pertussis* and that there are fitness advantages to certain genotypes under this pressure, specifically those genotypes that allow for vaccine escape (Belcher & Preston 2015). In this paper we use whole genome sequencing on a collection of isolates dating from 1935 (pre-vaccine era) to 2013 (current vaccination composition and schedule) to determine how selective pressures from vaccination may have influenced the evolution of *B. pertussis* with a specific focus on the USA.

5.3 Methods

We selected one hundred *B. pertussis* isolates from the CDC, FDA, and from a 2012 Washington outbreak for whole genome sequencing to better understand the effect of vaccination on the *B. pertussis* population. The isolates, dating from 1935-2013, were randomly selected by location and vaccination time period to try and maximize the diversity within our collection. We also included an oversample of isolates from the Washington 2012 outbreak to better understand diversity within a single outbreak.

5.3.1 Selection of Isolates

5.3.1.1 Selection of CDC isolates:

Isolates were selected from the CDC Schmidtke et al. collection (Schmidtke et al. 2012) based on unique year and state isolated combinations. Isolates of the same year and state are likely to be from the same outbreak and are likely to be highly clonal (Mooi 2010). This selection method identified 234 different isolates from 46 different

states plus the US Virgin Islands and St. Croix. An additional 23 isolates were randomly selected from the Washington 2012 outbreak collection (Table 5.2).

5.3.1.2 Selection of FDA isolates:

All available pertussis isolates with information on isolation date were selected from the FDA's collection of historic isolates. Analysis was limited to isolates that could be grown from the lyophilized samples, included information on date isolated, and were isolated in the United States (n=15) (Table 5.2).

5.3.1.3 Selection of MDCH isolates:

All available isolates from Michigan Department of Community Health (MDCH) were obtained (n=4) (Table 5.2).

5.3.1.4 Selection of Washington isolates:

The 2012 Washington outbreak collection contained 234 isolates. Ten percent of this collection (23 isolates) were randomly selected and included in this study (Table 5.2).

5.3.2 Sampling of isolates for whole genome sequencing:

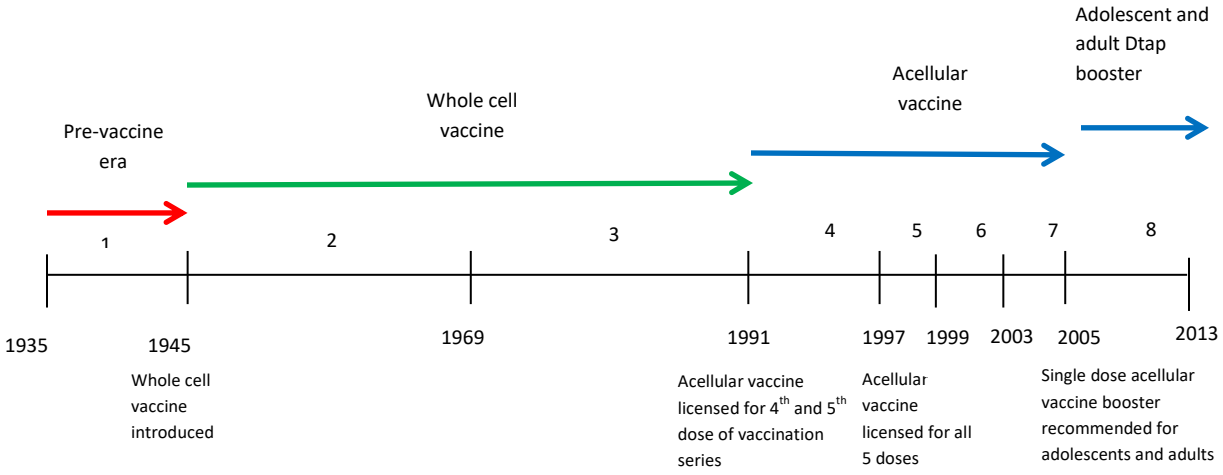
Funding was available to sequence 100 of the isolates. Our selection of isolates for sequencing was a follows. Isolates were first stratified by time period following the scheme proposed by Schmidtke et al: period 1 (pre-vaccine era) 1935-1945, period 2 (early whole cell vaccine era) 1946-1969, period 3 (late whole cell vaccine era) 1970-1990, period 4 (combination of whole cell vaccine and acellular vaccine for 4th and 5th doses) 1991-1996, period 5 (early acellular vaccine era) 1997-1999, period 6 (middle acellular vaccine era) 2000-2002, period 7 (late acellular vaccine era) 2003-2005, and period 8 (Tdap booster era) 2006-2013 (Figure 5.1) (Schmidtke et al. 2012). All five isolates from period 1 were sequenced (both FDA and CDC isolates). To reduce as much variation due to source as possible, we limited selection for the other time periods to isolates from CDC (including the WA isolates). Ten isolates were randomly selected from periods 2-7 to provide adequate coverage of all time periods. Thirty-five isolates were selected from period 8 for sequencing: 25 were a random sample from the time

period and an additional 10 were randomly selected from the Washington 2012 outbreak to allow us to answer questions about diversity within a single outbreak (Table 5.2). A larger sample from period 8 allows for more in depth study of this most recent time period.

Table 5.2 Vaccination time periods with number of isolates and source, 1953-2013

Time Period	Years	Vaccine Era	Number of Isolates					Selected for Sequencing
			CDC	FDA	MDCH	WA	Total	
1	1935-1945	Pre-vaccine era	3	2			5	5
2	1946-1969	Early whole cell vaccine era	16	2			18	10
3	1970-1990	Late whole cell vaccine era	42	11			53	10
4	1991-1996	Combination of whole cell vaccine and acellular vaccine for 4^{th} and 5^{th} doses	35				35	10
5	1997-1999	Early acellular vaccine era	45				47	10
6	2000-2002	Middle acellular vaccine era	34				34	10
7	2003-2005	Late acellular vaccine era	28				28	10
8	2006-2013	Tdap booster era	28		4	23	55	35

Figure 5.1 Timeline of vaccine introduction into the United States with key periods for changes in vaccine selection pressure



5.3.3 DNA extraction and purification:

DNA was extracted using a QIAGEN (Venlo, Netherlands) DNeasy Blood & Tissue kit and the QIAcube automated DNA extraction system. One hundred microliters of each sample was first incubated at 37°C with an 80 µL enzyme cocktail comprised of Promega cell lysis solution (Madison, WI, USA), lysozyme, mutanolysin, RNase A, and lysostaphin (Sigma Aldrich) in 22.5:4.5:1.125: 1.125:1 parts, respectively for 30 minutes. After extraction, DNA quantity and quality was measured using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA), a picogreen quantification assay, and agarose quantification against a Lambda HindIII ladder (New England Biolabs). Extracted DNA was stored at −80°C.

5.3.4 Library Preparation and Base Calling:

Library preparation (conversion of genomic DNA into sequencer-ready fragments) and high-throughput DNA sequencing was conducted by the Center for Microbial Genetics and Genomics at Northern Arizona University. Base calling and image analysis was completed using the Illumina System with at least 25X coverage.

5.3.5 Quality Control:

In addition to the quality control provided by the Illumina manufacturer, FastQValidator was applied to check for errors, establish minimum read length, and examine deviation from expected GC content and the average phred quality per cycle and overall average quality (http://genome.sph.umich.edu.proxy.lib.umich.edu/wiki/FastQValidator).

5.3.6 Alignment:

Reads were aligned against the *Bordetella pertussis* Tohama 1 genome (Parkhill et al. 2003) using the Burrows-Wheeler Aligner BWA-mem algorithm (available: http://bio-bwa.sourceforge.net/). The resulting .sam files were converted to .bam files then sorted and indexed using samtools (Li et al. 2009). The MarkDuplicates tool of Picard was used to identify and remove PCR optical duplicate reads originating from a single fragment of DNA that can occur during sample preparation (https://broadinstitute.github.io/picard/index.html). The MarkDuplicates tool was used with REMOVE_DUPLICATES=True, CREATE_INDEX=true, and VALIDATION_STRINGENCY=lenient. The Picard output was then indexed using Samtools (Li et al. 2009).

5.3.7 Variant calling and filtration:

Samtools mpileup (http://samtools.sourceforge.net/mpileup.shtml) was used to collect summary information from the input bam files. It calculates the likelihood of data given each possible genotype then stores the results in BCF format. The resulting BCF files were piped into Bcftools for variant calling (https://samtools.github.io/bcftools/bcftools.html#call). The following options were selected for samtools mpileup: -u (generates uncompressed output which is better for piping), -g (generates genotype likelihoods and output them bcf), and -f (indexed reference file is in FASTA format. The following options were selected for Bcftools call: -

Ov (output type uncompressed vcf), -v (output variant sites only), -c (consensus-caller method), -o (specify output file).

Variant filtering was conducted using the GATK -VariantFiltration tool (https://software.broadinstitute.org/gatk/guide/tooldocs/org_broadinstitute_gatk_tools_walkers_filters_VariantFiltration.php). We used the --filterExpression argument with FQ<0.025 (consensus quality - positive indicates heterozygote, negative indicates homozygote. In bacterial samples it provides evidence as to whether a particular gene was duplicated in the sample), MQ > 50 (Root mean square mapping quality is an estimate of the overall mapping quality of reads supporting a particular variant call), QUAL>100 (selects for phred-scaled quality score greater than 100), and DP>15 (requires more than 15 to support a variant call at a given position). Vcftools was used to remove indels and keep only the SNPs that passed the GATK filtering criteria (Danecek et al. 2011)

5.3.8 Generation of consensus sequences and multi-alignment FASTA file

After filtration and removal of indels, the VCF files were zipped and indexed with bzip (http://www.htslib.org/doc/tabix.html) and bcftools (https://samtools.github.io/bcftools/bcftools.html#index), respectively. A consensus sequence for each *B. pertussis* isolate was generated by applying the filtered VCF files to the *B. pertussis* Tohama 1 reference FASTA files with the use of bcftools consensus (https://samtools.github.io/bcftools/bcftools.html#consensus). Once the consensus sequences were generated, the headers were changed to reflect the isolate name and date rather than the referent name. The consensus sequences were then concatenated to generate a single multi-alignment FASTA file. Snp-sites was used to extract SNPs from the multi-alignment file (Keane et al. 2016)

5.3.9 Annotation and Analysis

SNPs were annotated using script provided by Yancy Lo. A neighbor joining tree with 100 bootstrap replicates was created using Seaview. Invariable sites and across

site variation were both set to “none”. Default parameters were used for Tree searching operations and Starting tree (Gouy et al. 2010). The tree was then uploaded into iTOL, midpoint rooted, and color-coded by vaccine era (Letunic & Bork 2016).

A pairwise distance matrix was created with the ape package in R (http://ape-package.ird.fr/). Histograms of SNP locations and pairwise distances were also created using R. Further analysis of branch length and pairwise distance by vaccination period was conducted using SAS software version 9.4 (SAS Institute, Cary, NC). The corr procedure was used to assess correlation between branch length and isolation date and pairwise distance from FDA-2 and isolation date. ANOVA was used to detect overall differences in branch length or pairwise distance by vaccination period and the tukey test to assess specifically which vaccination periods differed from one another. Analyses were conducted with and without the FDA-2 isolate as it was an outlier in the analyses and strongly influenced the results.

This project (HUM00052197) received exempt status from the University of Michigan Institutional Review Board on June 27, 2011.

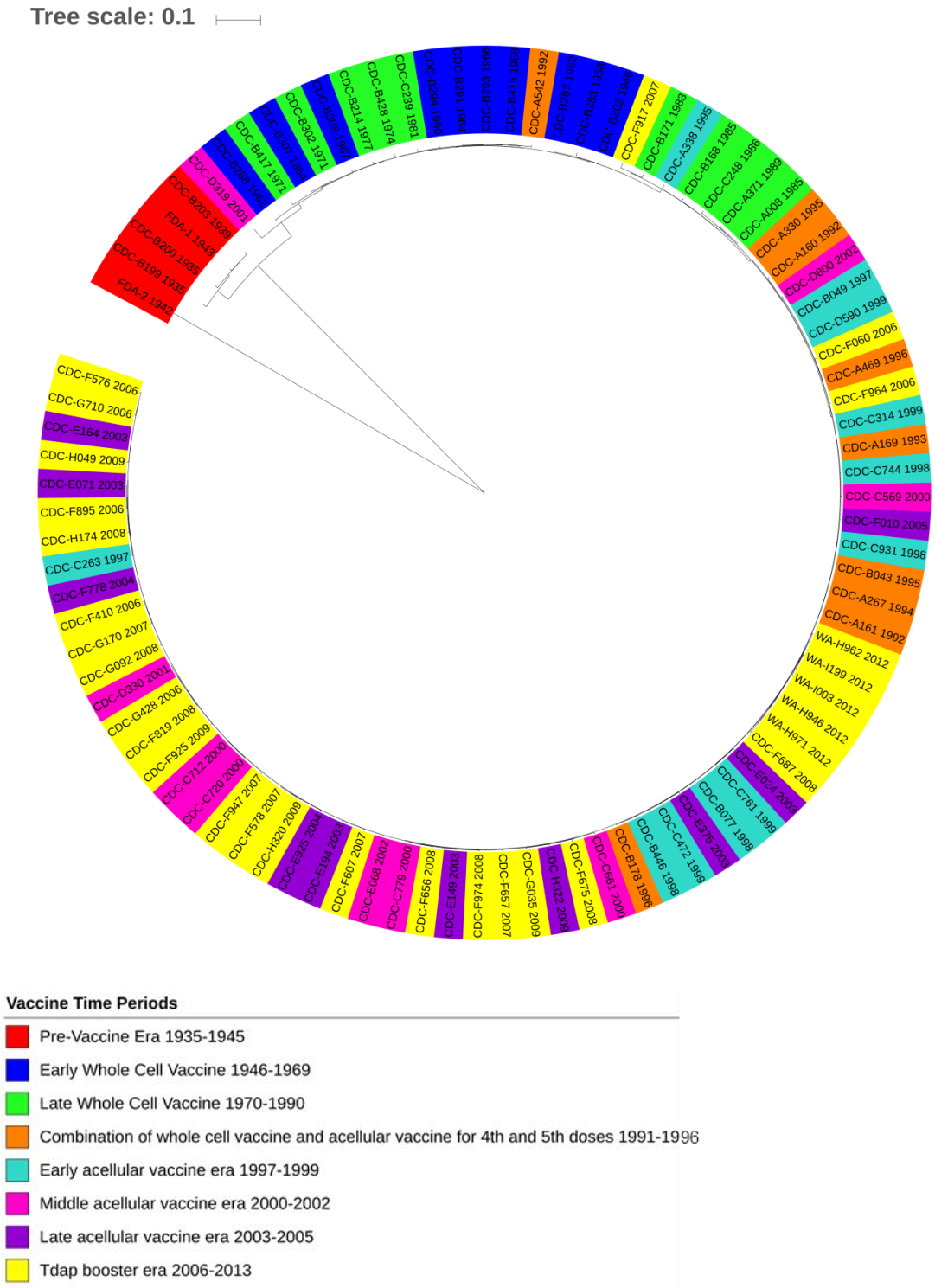
5.4 Preliminary Results

Of the 100 *B. pertussis* isolates sent for whole genome sequences, 93 produced high-quality sequences that could be used in the analyses. One isolated from period 6 (Middle Acellular Vaccine Era), 1 isolate from period 7 (late acellular vaccine era), and 5 isolates from period 8 (Tdap booster era) were excluded from the analysis due to absence of usable sequence data.

The phylogeny revealed 2 main clades similar to the phylogeny produced by a global collection of 343 strains isolated between 1920 and 2010 (Figure 5.3) (Marieke J. Bart et al. 2014). There was a distinct separation between the pre-vaccine era isolates and the rest of the collection ($p<0.0001$). These results support a significant effect of vaccination on extinction of strains resulting in a replacement of populations.

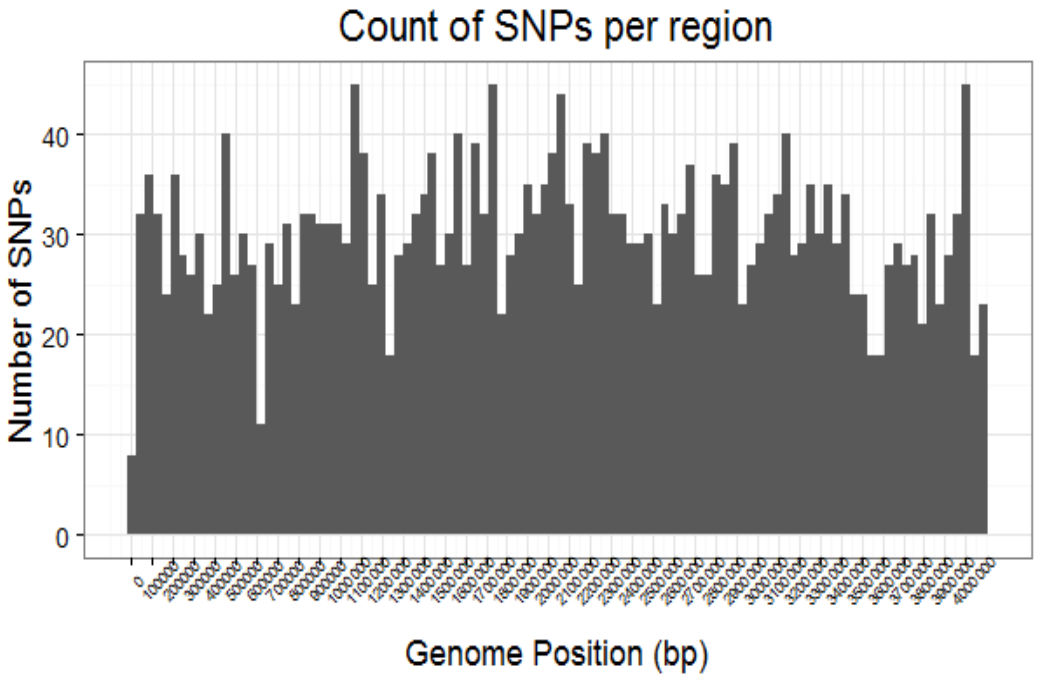
Many of the smaller clades had low bootstrap values indicating little support for evolutionary relationships between strains. All pre-vaccine strains were in clades that contained no post-vaccine strains.

Figure 5.2 Neighbor-joining tree with 100 bootstrap replicates. Color shading represents vaccine time period. The branch length depicted in the legend represents an average of 0.1 nucleotide substitutions per site.



We found 3,018 SNPs between this collection and the Tohama 1 reference genome. Similarly Bart et al. found limited genetic diversity in their collection, identifying only 5414 SNPs and an average SNP density of 0.0013 SNPs/bp (Marieke J. Bart et al. 2014). There did not appear to be any hot spots in the genome for substitution (Figure 5.3).

Figure 5.3 Count of SNPS per genome region from multi-sequence alignment



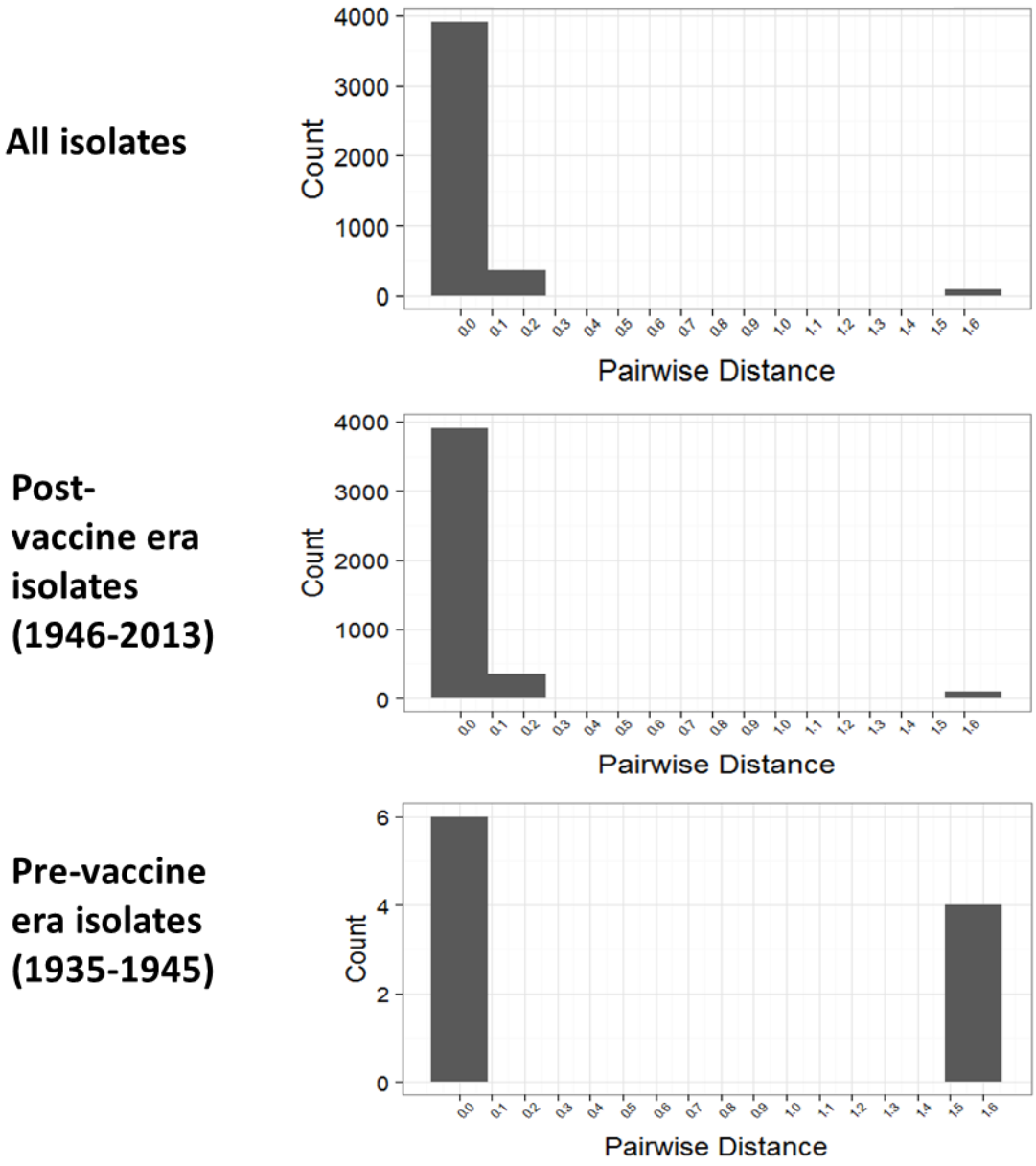
Since the introduction of vaccination, the diversity between strains has reduced. The average pairwise difference between strains during the pre-vaccine era was 0.01 (range: 0.01-0.02) and decreased to an average of 0.0007 (range: 0.0007-0.1) after the introduction of vaccination indicating a smaller population size. Vaccination likely created a genetic bottleneck, where a very small subset of the population survived. This is contrary to findings in a global collection of *B. pertussis* isolates which found no loss in diversity following the introduction of vaccination (Marieke J. Bart et al. 2014).

However, studies in more globally restricted populations, such as ours, also observed a decrease in population diversity after introduction of vaccination as would be expected if selective pressure from vaccination resulted in a population bottleneck where strains immune to the vaccine are more likely to survive (Litt et al. 2009; Van Loo & Mooi 2002; Weber et al. 2001).

In the histogram of pairwise distances for all isolates, there are three bars; the largest is at the smallest pairwise distance because the post-vaccine era isolates are so similar to one another (Figure 5.4). The second largest bar was also at a very small pairwise difference, because although the pre-vaccine era isolates were distinctly clustered from the post-vaccine era isolates they were still highly similar. The largest pairwise differences were the distances of all the isolates from FDA-2, a pre-vaccine era isolate.

Additional analyses, as outlined below, are needed to explore the effect of acellular vaccination on the *B. pertussis* population, characterize diversity within a single outbreak, and to explore the possibility of asymptomatic carriage as a major reservoir of pertussis infection.

Figure 5.4 Count of isolates for each pairwise distance by pre-vaccine and post-vaccine era



5.5 Future Analyses

5.5.1 Re-align to closed genome to explore genome rearrangement in our strains

The first task will be to re-align the reads from our strains to a closed, ordered genome that was created using a combination of long-read sequencing and restriction optical mapping. This will give us a better picture of the presentation and variation in location of insertion sequence elements. The majority of *B. pertussis* sequences generated so far were created with the use of Illumina short-read sequencing, which creates reads that are not long enough to span the 1kb IS481 repeat regions making it impossible to create a closed, ordered genome sequence from the data (Belcher & Preston 2015). With the introduction of long-read sequence platforms, it is now possible to generate long-range scaffolds and create closed genome sequences (Liao et al. 2015). A study of 31 statewide epidemic strains and 2 re-sequenced vaccine strains combined short- and long-read sequencing platforms with restriction optical mapping to create a closed genome de novo assembly. Using this method, they were able to identify 16 distinct genome rearrangement profiles among the epidemic strains, all of which were distinct from the vaccine isolates (Bowden et al. 2016). More copies of IS481 were also identified in the majority of epidemic strains as compared with the vaccine strains indicating that gene inactivation by insertion of IS481 may be an important method for allowing *B. pertussis* to evade acellular vaccine-induced immunity in the host (Bowden et al. 2016). As further evidence, an IS481 was found within the prn gene locus in the majority of prn-deficient strains in the U.S. (Pawloski et al. 2014; Bowden et al. 2014). Closed genomes were also assembled for two Dutch strains with three large inversions identified between the strains suggesting the potential for extensive variation in genome arrangement among *B. pertussis* strains (Marieke J Bart et al. 2014; Belcher & Preston 2015). Given the apparent importance of genome rearrangement as a source of variation between *B. pertussis* strains, alignment to a closed genome will allow us to explore this in our strains. For example, if we identify prn-deficiency in our strains, is it due to an insertion of IS481?

5.5.2 Identify temporal trends in strain frequencies for vaccine antigen alleles

Studies worldwide document divergence between vaccine strains and currently circulating strains with respect to the antigens contained in the acellular vaccines (Schmidtke et al. 2012; Cassiday et al. 2000; Kallonen & He 2009; Octavia et al. 2012; Advani et al. 2011; Bart et al. 2010; Borisova et al. 2007; Mooi et al. 1998). To assess how vaccination may have affected our *B. pertussis* population, we will assess the strain frequencies for each of the acellular vaccine antigen (*fim2, fim3, ptxA, ptxP, prn*) alleles by the eight vaccination time periods outlined. This will allow us to determine if any allele shifts over time, such as the *ptxP1* to *ptxP3* identified by Bart et al. can be observed in our collection (Marieke J. Bart et al. 2014).

5.5.3 Further Characterize Identified SNPs and Calculate SNP Densities and Mutation Rates in Regions of Interest

We will classify the SNPs identified as non-coding, synonymous, or non-synonymous. A synonymous SNP is one that changes the codon to a different codon which codes for the same amino acid while a non-synonymous SNP codes for a different amino acid. Exploring the synonymous versus non-synonymous mutation rate in regions of interest, such as the antigen encoding genes, will provide information as to whether selection is acting on that particular region. The non-synonymous mutations can be further explored by identifying the functional classification of the predicted protein categories using a database of functional annotation such as EggNOG 4.5 (Huerta-Cepas et al. 2016). Studies in a global collection of *B. pertussis* isolates and a collection of isolates from two statewide outbreaks in the United States both found an overrepresentation of non-synonymous SNPS in transport proteins which may suggest this as a method of adaption to vaccination (Marieke J. Bart et al. 2014; Bowden et al. 2016). Whether particular gene categories are evolving faster than others can be explored by determining if the SNP densities in these regions of interest differ significantly from the overall SNP density of the genome as a whole. SNP densities in the vaccine antigen-encoding genes will also be compared between eras to determine if

selection pressure has increased on these regions since the introduction of acellular vaccines.

5.5.4 Further Study Outbreak Strains to Characterize Diversity Within a Single Outbreak

In a study of 100 United Kingdom *B. pertussis* isolates dating from 1920-2012, with particular emphasis on isolates from the 2012 outbreak, Sealey et al. showed that many distinct strains contributed to the 2012 outbreak and that it was not due to the emergence of a novel and more virulent clone or to the expansion of an individual lineage (Sealey et al. 2015). They also found very little difference between outbreak strains and strains isolated during periods of low pertussis incidence (Sealey et al. 2015). Further analyses of our outbreak strains are needed to determine if there is evidence of clonal spread of an individual lineage or novel clone within the outbreak or if many strains contributed.

5.5.5 Determine Whether Our Population of Strains Support Asymptomatic Cases as a Significant Reservoir for Infection

In an analysis of 36 U.S. *B. pertussis* clinical isolates, Althouse and Scarpino found more genetic diversity in the bacterial population than could be explained by the observed number of infections without the possibility of asymptomatic carriage and that the time of changes in age-specific attack rates were consistent with asymptomatic transmission (Althouse & Scarpino 2015). Seroprevalence studies suggest that many more people are infected with *B. pertussis* than actually present with disease (HUYGEN et al. 2014; RØNN et al. 2014; Scott et al. 2015). These studies suggest that *B. pertussis* is endemic in the population, but only causes disease in a fraction of those infected (Belcher & Preston 2015). Preliminary analysis of our isolates suggests limited asymptomatic carriage in the pre-vaccine era although further analysis is needed to confirm this and to assess whether there is evidence to support a larger role of asymptomatic carriage since the introduction of the acellular vaccine. Although all of

our isolates were recovered from symptomatic individuals, it would be interesting to obtain isolates from asymptomatic individuals to determine if they represent a separate lineage (Belcher & Preston 2015).

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Chapter 6. Summary and Conclusions

6.1 What I Learned From 5.5 Years as a PhD Student

6.1.1 Collecting Data and Samples Takes Time and Lots of It

For this study, I gathered 275 *B. pertussis* isolates dating from 1935-2012, ten years of pertussis case report data from the California Department of Public Health, and case report data from Marin County Health and Human Services. The isolates took over two years to obtain and came from the Food and Drug Administration (FDA), Centers for Disease and Prevention (CDC), Michigan Department of Community Health (MDCH), and Seattle Children's Hospital.

The FDA collection came about when my Co-Adviser ran into an FDA pertussis researcher at a conference who offered access to his collection. Never underestimate the power of networking. After months of emailing back and forth, I was finally granted access to drive out to Bethesda and dig through the samples in their freezers myself to select what I wanted. As an interesting side note, I also learned how to ship infectious agents on dry ice. The isolates from the CDC proved to be the most challenging to obtain and the ones nobody thought could be obtained. From the time I first identified the collection in a journal article and contacted the author to when the isolates finally arrived in the lab, over two years had passed. On my part, it took persuasion, a proposal demonstrating that I had a good idea and the right people working with me to carry out the project, authorship agreements, and a few precious, pre-vaccine era samples that were not yet a part of their collection. It also took months of back-and-forth between the legal departments at both institutions to work out the material transfer agreements. The other samples were a little easier; my contacts at the CDC connected me to a

contact at Seattle Children's Hospital and my adviser connected me with a colleague and friend at MDCH who agreed to provide samples. In between these successes, there were plenty of failures. Not everyone I contacted was equally helpful or willing to share data and samples; however, most people were willing to help a genuinely interested and enthusiastic graduate student.

As a Marin County Epidemiologist before I returned to graduate school, I was able to obtain permission from the Public Health Officer to bring case report data with me for use on my dissertation. The data from CDPH was a little more challenging to obtain and involved over a year of back-and-forth emails and phone calls, and IRB applications at both institutions. It was important to keep in mind that these data requests create work for other people and that they also put considerable effort into the initial collection of data and samples. Being gracious yet persistent with requests and offering acknowledgement of the work of others through promises of authorship was instrumental in getting the job done. The most important lesson learned from this was to not underestimate the amount of time and effort required to find and obtain the appropriate data and specimens, and to not be afraid to reach out to other researchers in the field. Most were happy to help out a graduate student.

6.1.2 Projects Fail - Know When to Move On

I had great plans for the California case report data. I was going to examine how community factors such as vaccination rates, health care utilization, poverty level, and education affected an individual's probability of being infected. I spent a great deal of effort merging the California data with data from the US Census, California Department of Finance, California Expanded California Kindergarten Retrospective Survey of vaccination status, and California Vital Statistics. I excitedly analyzed the data according to my plan and sought advice from other committee members and colleagues on an appropriate analytic strategy. The problem was CDPH only releases data at the County level to protect the privacy of those infected. There was too much variability within a single county to identify community factors that predicted pertussis infection. I needed

geographically higher resolution data such as census tract level or smaller that would never be released to me. It was difficult to fathom dropping a project that was supposed to be a significant portion of my dissertation and that took so much effort and time to set up. My adviser matter-of-factly told me to cut bait and come up with an idea for a new project. She was right; it was a waste to throw more time and energy at a project that wasn't going to work.

Another failed project involved screening archival throat swabs from healthy individuals and individuals with influenza-like illness. From everything I'd read on pertussis, I knew testing was typically done using nasopharyngeal swabs. There might not be enough *B. pertussis* DNA in the throat to actually be detected. If we found *B. pertussis*, it would not only be great to have a positive result but also good to know that it can be picked up with a throat swab rather than the slightly more invasive nasopharyngeal swab. If we didn't find *B. pertussis*, we wouldn't know if it was because it truly wasn't there or if we were simply unable to detect it. I extracted the DNA, learned how to do qPCR, and started screening my samples. After screening about half of the samples, I had not found any positives. I quickly realized that the results would be meaningless. Zero positive samples didn't tell us anything, either none of the samples contained *B. pertussis* or we couldn't detect it from the throat swabs. I learned new laboratory skills in the process, but there was no use continuing with the project.

6.1.3 Biological Variability Exists - Challenges in the Lab

Outside of specimen and data collection, the project that took up the most amount of time was creating a high-throughput method for assessing for coaggregation among bacterial species and applying this method to the study of *B. pertussis* interactions with nasopharyngeal commensals. This was my first hands on experience with true biological variability. I would see exciting results of coaggregation between *B. pertussis* and a commensal strain one day, and then nothing the next. The same thing would even happen with my positive control strains that were well-documented in the literature as coaggregating strains. I experimented with changing the media, growth

times, and pH. I swapped coaggregation buffer for PCR-grade water and swapped the bacterial stock I was using for stock that had been passaged fewer times. After more than 30 trials on some of my crosses, I could not figure out why sometimes my coaggregation assays would work and sometimes they didn't. After some persuasion from my adviser, I finally presented my results at lab meeting. No one was surprised by my inability to get consistent results and everyone was wondering why I kept repeating my experiments determined to do so. It turns out that the species I was working with change phases and are known for being highly variable. Presenting my results earlier would have saved a lot of time and frustration. I was too concerned about having a completely finished project before presenting the results when I should have been seeking input from my colleagues throughout the project. Luckily, experiencing all of the variability helped me design a high-throughput methodology that allows for multiple replicates of a particular pair under identical conditions so outside factors that influence coaggregation can be further studied.

6.1.4 Working with Archival Specimens

I was excited to obtain a collection of nasopharyngeal swabs collected from healthy individuals that were collected as part of a former student's dissertation work on *S. pneumoniae* and felt they would be perfect for coming up with a prevalence estimate of asymptomatic *B. pertussis* carriage in a population highly vaccinated with the whole cell vaccine. Realizing that the samples were nearly a decade old and that there were no guarantees as to their current quality, I selected a random sample of isolates that the former student had identified as positive for *S. pneumoniae* based on culture and tried to duplicate her results using PCR. Out of the ten presumptive positive samples that I screened, nine resulted in positive determination giving me the false impression that the rest of the samples would be viable. I went ahead and extracted DNA from each of the samples and carried on with my experiment. I screened all of the samples for *B. pertussis* and got halfway through screening for *S. aureus*, when it became obvious that I was obtaining prevalence estimates for *S. aureus* (zero positive

samples) that were not even close to estimates from the literature of 19.0% to 55.1% (Kluytmans et al. 1997). It was clear that there was an issue with the archival samples. To prevent the wasted time, I should have put more time up front in screening more samples and made sure to screen samples from every freezer box. Working with archival specimens can be highly informative as new project ideas and technologies come to light, but it extremely important to thoroughly screen the samples for quality and viability.

6.1.5 It Takes a Village

During my final year of graduate school, I finally learned the importance of taking on undergraduate students to help with my projects. Once I developed a particular repetitive aspect of my protocol, such as DNA extraction, PCR, or coaggregation assays, it made way more sense to train an undergraduate researcher to help with the work rather than to try and do it all myself. I was able to develop a protocol and learn the skills necessary to carry it out, a student had the benefit of gaining research experience, and the project was completed much faster. Providing training, delegating tasks, and overseeing the work of others are all important skills gained from allowing undergraduate researches to help with projects.

Seek input early on and throughout a project. Presenting protocols while still in development and preliminary results can improve projects, get the work done faster, and might prevent failed projects. Rather than re-inventing the wheel, seek guidance from your committee and other researchers in the field. It's not cheating, it's efficient.

6.1.6 Gaining an Incredible Skill Set

While some of my projects failed and others I wasn't able to complete as much as I'd have liked too, I still gained an incredible skill set. I learned laboratory skills such as DNA extraction, PCR, qPCR, and how to carry out a Picogreen DNA quantification assay. I learned how to develop and troubleshoot protocols and work with numerous types of laboratory equipment such as a confocal microscope and a FlowCam device. I

became competent in working with many different bacterial species. Most importantly, I became competent and confident in learning new techniques and where and how to search for answers to my questions.

Outside of the lab, I became comfortable developing research questions and setting up a study that could answer those questions. I could identify potential sources of bias and think of ways a project could be improved. I became skilled at creating protocols that could obtain clear and consistent results between researchers and learned to develop a manuscript for a peer-reviewed journal.

For my genomics project, I was fortunate to be selected to attend a Working with Pathogen Genomes Workshop at the Wellcome Trust Sanger Institute. Here I learned to use Unix and work with programs from the command line, map and assemble genetic sequence data, call SNPs and identify regions of difference between genomes, annotate genomes, and develop phylogenetic trees. Working with big data, learning and troubleshooting new software, and blending software programs together to create a pipeline that carried out all of the necessary tasks proved to be extremely difficult, but I did it. Unfortunately the learning curve on this project was so steep, I barely scratched the surface of the analyses I'd planned to do. I was able to create a pipeline that worked, create a tree, and conduct some preliminary analysis. I walked away with these skills and a better understanding of how genetic sequence data can be used to answer questions of epidemiologic importance.

Outside of my dissertation work, I was also given the opportunity to teach. I gained experience in developing lesson plans, assignments, and tests; public speaking, working with a diverse group of students, and tailoring my instruction to meet the needs of my students.

6.2 Outstanding Issues and Future Directions for My *B. pertussis* Work

6.2.1 High-throughput Method for Assessing Coaggregation Among Bacterial Species (Chapter 2)

Combining the high-throughput screening method with confocal microscopy and the FlowCam device opens the door for in-depth studies of aggregation and coaggregation among large panels of test strains. These methods can be used to identify the factors that contribute to high variability in coaggregation assay and to determine if coaggregation occurs in other biological systems.

6.2.2 Coaggregation between *B. pertussis* and NP commensals (Chapter 3)

Further study is needed to better understand how *B. pertussis* interacts with nasopharyngeal microbiota. While we provided evidence that some interaction is occurring, we were unable to clearly identify the type of interaction.

A case-control study in which nasopharyngeal swabs are taken from those with and without *B. pertussis* would be useful to identify differences in the microbiota between the two groups. DNA from the swabs could be extracted and sequenced to give an idea of community composition (Foxman & Rosenthal 2013). The swabs could also be cultured to capture the specific strains that frequently co-occur with one another and ones that never do. Our research on *B. pertussis* showed there can be considerable strain variation in type and extent of interactions observed.

Further studies could be done to assess for coaggregation or interbacterial competition between strains. *B. pertussis* contains several mechanisms, such as bacteriophages and a type IV secretion system for secreting pertussis toxin, which may be important for interaction with the host microbiota and the ability to colonize a host (Weyrich et al. 2014). Understanding the interaction between *B. pertussis* and the host microbiota may have implications for control and treatment. Weyrich et al. point out that susceptibility to colonization by *B. pertussis* may be increased by disruption to the resident microbiota of the nasal cavity and hypothesize that this may contribute to why

infants, without a mature nasal cavity microbiota, are more susceptible to pertussis infections than healthy adults (Weyrich et al. 2014). If the microbiota is important for fighting off *B. pertussis* colonization, the administration of broad-spectrum antibiotics could potentially increase risk colonization and disease among infants (Weyrich et al. 2014).

6.2.3 Estimation of *B. pertussis* asymptomatic carriage rates and co-occurrence with NP commensals (Chapter 4)

Understanding the prevalence of asymptomatic carriage is important for determining the role it plays in the transmission system. A study of nasopharyngeal *B. pertussis* carriage in healthy people is warranted – although getting healthy people to submit to an NP swab poses a challenge. DNA could be extracted from these swabs and sequenced to identify organisms present. This would be much more efficient than using PCR to screen for selected organisms and would require no a priori list of organisms of interest. As this method often can't resolve below the genera level, a mix of untargeted and targeted screening would likely be necessary.

6.2.4 Evolution of *B. pertussis* in response to vaccination (Chapter 5)

We have barely scratched the surface in terms of what can be done with the *B. pertussis* sequences. As the samples for sequencing included an oversample of isolates from a single 2010 outbreak in Seattle, Washington, we can further explore these sequences to better understand diversity within a single outbreak. Additionally, given that we have an external clock (1935-2013) based on the isolation dates, we can estimate the molecular clock for *B. pertussis.* The SNP annotation we created can be used to determine the presence of positive selection on gene regions throughout the genome and to identify gene regions that are highly conserved. We can also determine if allele frequencies differ in gene regions that code for the antigens commonly found in vaccines compared with other gene regions to see if selective pressures differ by gene region. Other regions of interest include the gene regions that code for adenylate cyclase, lipopolysaccharide endotoxin, dermonecrotic heat-labile toxin, tracheal cytoxin,

the bvgAS locus, and various serotype specific agglutinogens and envelope proteins that may be important for virulence or immune escape and have demonstrated sequence variation (Packard 2004; Bart et al. 2010).

Mooi et al. suggest that vaccination may have selected for strains that can transmit through vaccinated hosts more efficiently by delaying the immune response; vaccines effectively remove infants as a major factor in transmission, changing the ecology of *B. pertussis*. We can explore this hypothesis by determining if any of the mutations we have identified could be involved in immune suppression (Mooi 2010). We can also determine how phylogenetic trees based on SNPs identified from whole genome sequences align with currently published predictions of *B. pertussis* genome evolution based on MLST patterns. We can also compare the results of our analyses from mapping to the Tohama 1 reference genome with those from a de novo assembly and to a more recently published closed genome created with PacBio.

6.3 Implications for pertussis intervention

In this dissertation we explored several mechanisms that may contribute to resurgence of *B. pertussis* in the United States: interaction with nasopharyngeal microbiota, asymptomatic carriage, and divergence of circulating strains from vaccine strains. Understanding how *B. pertussis* interacts with the host microbiota may give insight into the sources of variation in risk of colonization given exposure, particularly considering that so many pertussis cases occur in fully vaccinated individuals. It may also explain why infants with an underdeveloped microbiota are more susceptible to infection (Weyrich et al. 2014). Given recent evidence that acellular vaccines may protect against disease but not against colonization, a better understanding of the actual prevalence of asymptomatic carriage is important for assessing its role in transmission and for decision-making regarding pertussis control. Lastly, understanding how *B. pertussis* evolves in response to vaccination is essential for developing vaccines with the most effective components and determining the best vaccination strategies.

6.4 References

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