THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

A STUDY OF THE GENES REQUIRED FOR *BORDETELLA BRONCHISEPTICA* TRANSMISSION AND BORDETELLAE'S INTERACTION WITH *DICTYOSTELIUM DISCOIDEUM*

LINDSAY HILBURGER
FALL 2016

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Veterinary and Biomedical Sciences
with honors in Veterinary and Biomedical Sciences

Reviewed and approved* by the following:

Eric Harvill
Adjunct Professor of Immunology and Infectious Diseases
Thesis Supervisor

Lester Griel
Professor and Program Coordinator of Veterinary and Biomedical Sciences
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Bordetella species are respiratory pathogens that are important in both human and veterinary medicine, with the ability to infect and transmit amongst and between multiple mammalian hosts. Causing millions of infections worldwide each year, current *Bordetella* research is focused on understanding and preventing transmission between susceptible individuals. It has long been thought that *Bordetella* species are only capable of transmitting from host to host via the direct transmission of aerosol droplets. However, recent studies have shown the capability of *Bordetella bronchiseptica* to survive intracellularly within the amoeba species *Dictyostellium discoideum*. These studies indicate that amoeba may represent an environmental reservoir for *Bordetella*, as a mode of extracellular survival permitting transmission. In the first chapter of this thesis, we show that not just *B. bronchiseptica*, but other species of *Bordetella*, including both classical and non-classical species, are also capable of this complex interaction with amoeba. Additionally, to better understand the classical direct process of transmission between live hosts, we developed conditions to study *B. bronchispetica* transmission between mice. The second chapter of this thesis aims to determine the virulence factors necessary for colonization, shedding, and transmission of *B. bronchiseptica* from infected hosts to exposed mice. In this chapter, we show that capsule gene is required for the transmission of *B.bronchiseptica* in mice. From this study, we can highlight more effective vaccine targets for both humans and animals, and overall shed light on ways to help limit the spread of *Bordetella* from host to host.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

There are many to thank for making my 3 and a half years at Penn State some of the most intellectually enriching of my life. First and foremost, to Eric Harvill and the entire Harvill lab, I am eternally grateful for your mentorship and contribution to my development as an undergraduate researcher. I wish you all the best at University of Georgia. To Eric, you have provided me with opportunities only dreamed of by most undergraduates, and your faith in me as a scholar, student, and future scientist has been a huge encouragement to me. To Dawn Taylor, my science mom, thank you for many hours of commitment to my projects. You have taken countless hours out of your day to edit, plan experiments with me, and troubleshoot when things go wrong. I am happy to have gotten to know you on a more personal level. (Thanks for watching my cat that one time.) Holly, Israel, Hamidou, and Kalyan, I appreciate your mentorship, guidance, and friendship. I could always count on you for a good laugh or intriguing conversation, even when I was having a difficult day. I am honored to have been able to work with you. To Liron Bendor, thank you for trusting me with your graduate thesis, and for taking me in as a sophomore with virtually no experience. Though our time together in the lab was short, I am endlessly grateful for the opportunities you have given me, and without your work, my thesis would be non-existent. To Shannon and Emily, thank you for completing our "wonder-trio." As a fellow undergraduate in the lab, I wish you all the best in your futures, I know they will be bright. To my advisor and Professor, Dr. Griel, thank you for your guidance over the years. I have honestly found your classes some of the most interesting, and will undoubtedly carry what I have learned from you (and your memorable stories!) to vet school and beyond.

Background

Bordetella are gram-negative coccobacilli bacteria capable of causing respiratory infections in humans and other mammals. The most commonly studied species are the classical *Bordetella* species: *Bordetellae pertussis, Bordetella parapertussis,* and *Bordetella bronchiseptica*. Important pathogens relevant to both human and veterinary medicine, *B. pertussis* is the causative agent of whooping cough in humans while *B. bronchiseptica* causes kennel cough in dogs and atrophic rhinitis in swine.

Within human populations, *B. pertussis* infections cause an estimated 294,000 deaths worldwide each year (Paddock et al., 2008). Most deaths occur primarily among young children, with infants being the most susceptible to infection. *B. pertussis* can cause life-threating complications in these children, such as dehydration, anorexia, apnea, pneumonia, seizures, and even death in severe cases (Centers for Disease Control and Prevention, 2015). *Bordetellae* colonize ciliated cells of the respiratory mucosa, particularly in the trachea and bronchi (Finger and Von Koenig, 1996). The clinical manifestation of whooping cough is rather extensive, with symptoms often persisting for months. After a 1 to 2 week incubation period, the disease begins with the catarrhal phase, characterized by symptoms of low-grade fever, progressive cough, and nasal discharge (Centers for Disease Control and Prevention, 2015). The disease then progresses to the paroxysmal stage, depicted by the pathognomonic inspiratory "whoop" followed by uncontrollable coughing fits. Paroxysms are often violent and long lasting, leading to vomiting and cyanosis. This stage can persist for 2-6 weeks or longer. The third stage, or the convalescent

stage, is characterized by gradual recovery and lessening severity of paroxysms (Centers for Disease Control and Prevention, 2015). Importantly, the insidious nature of whooping cough adds to the severity of the disease. Specifically, *B. pertussis* is capable of asymptomatic transmission during the clinical manifestation's incubation period, as well as at any point between asymptomatic carriers of the disease (Althouse and Scarpino, 2015).

Fortunately, a vaccine does exist for *B. pertussis*. However, despite sound vaccination programs (and thus high vaccine coverage) , a recent resurgence of yearly *Bordetella* infections has been observed in the United States, Australia, and other developed countries. Most notably, in 2012, the United States reported more diagnosed *B. pertussis* cases than in any year since the introduction of the *Bordetella* vaccine in 1955 (Althouse and Scarpino, 2015). *B. pertussis* has since has adapted to evade the immune systems of vaccinated populations to both infect and transmit between individuals. The first whooping cough vaccines, whole-cell vaccines (WCV), were the first to be used in the 1950's. However, due to intense adverse reactions in babies and children, the WCV was replaced by acellular vaccines (ACV) in the 1980s and has since continued to be used in developed countries (Lam et al., 2014). The current ACV vaccine targets only a few critical *B. pertussis* antigens, and the majority of ACV formulations target the following virulence factors: pertussis toxin (*ptx*), pertactin (*prn*), and filamentous hemagglutinin (*fha*) (Centers for Disease Control and Prevention, 2015). Shockingly, recent studies have reported increasing isolation of *B. petussis* lacking expression of pertussis toxin and pertactin, leading to speculation of genotypic adaptation and resistance to the current vaccine (Bouchez et al,. 2009)(Otsuka et al., 2012). These reports, along with the increasing incidence rates have called for a more thorough investigation of the mechanisms behind *Bordetella* spp. transmission, particularly the effectiveness of current vaccines.

Mouse Model of Bordetella Transmission

The success of *B. pertussis* as a human pathogen (and the recent re-emergence of the disease) has lead to the increasing interest in the study of underlying mechanisms of infection and transmission with *in vivo* models. Although the baboon model most closely mimics human infection by *B. pertussis* and therefore successfully models whooping cough, it is costly to maintain and raises ethical concerns (Warfel et al,. 2012). *B. bronchiseptica* is naturally capable of naturally infecting a variety of mammalian hosts including swine, which has also been a useful model to study *Bordetella* transmission (Nicholson et al,. 2012). Despite this, both swine and baboons lack the immunological potential of transgenic mice, as well as the low cost and maintenance of the mouse model. Previously, our lab has established an experimental model in which to study transmission in mice (Smallridge et al,. 2014). Furthermore, *B. bronchiseptica* and *B. pertussis* genes are highly conserved, including but not limited to the factors pertactin (*prn*) and filamentous hemagluttinin (*fha*). This high rate of conservation allows for an efficient means to identify genetic candidates in *B. bronchiseptica* for further study in the more costly primate systems with *B. pertussis*.

Bordetella's Interaction with Dictyostelium discoideum

Until recently, the prominent consensus is that *Bordetella* species are only capable of transmitting from host to host via the direct transmission of aerosol droplets. However, the discovery of intracellular survival of *Bordetella spp.* in human macrophages, and thus their avoidance of phagocytosis, suggests that *Bordetella spp.* may also be capable of surviving intracellularly within other eukaryotic cells (Lamberti et al., 2010). *B. bronchiseptica* is also

believed to survive in the environment via the up-regulation of genes that increase motility and the ability to survive nutrient deprivation. (Bendor et al,. 2016). Despite this, an environmental reservoir for *Bordetella* remains only speculative.

Dictyostelium discoideum is a soil-dwelling amoeba that feeds primarily on bacteria. They are most commonly found in the leaf litter of tropical to temperate forests, where bacterial food sources are plentiful. (Swanson et al,. 1999). *D. discoideum* has been used as a model organism for the past 50 years, particularly for the understanding of cellular motility, signaling and interaction (Eichinger et al,. 2005). Its asexual life cycle is unique, consisting of single and multicellular stages determined by availability of food sources. When food resources (bacteria) are plentiful, *D. discoideum* survives as single-celled trophozoites. During times of environmental stress, these amoeba are capable of aggregating together to first form a multi-cellular slug. Ultimately, the aggregation results in a fruiting body, consisting of a stalk and spore-containing sori. These sori can then become dislodged and dispersed with the goal of spreading to new food sources (Bendor et al., 2016).

Although many bacteria are simply food sources for amoeba such as *D. discoideum*, some other bacterial species such as *B. bronchiseptica* have previously been shown to be capable of avoiding predation by these amoeba and surviving intracellularly within the sori. Furthermore, *B. bronchiseptica* does not only survive amoebic predation, but can propagate within the sori of *D. discoideum*, transferring it to new locations. *B. brochiseptica* present in sori are also capable of geographical spread by flies, and bacteria persisting in amoeba are able to effectively infect lab mice, indicating that amoeba may be a potential environmental reservoir and ultimately an important vector in transmission (Bendor et al,. 2016).

Preface

This thesis aims to investigate transmission dynamics of classical and non-classical *Bordetella* species, particularly *B. bronchiseptica,* to better understand how to disrupt transmission and prevent the spread of illness. In the first chapter of this thesis, we observed the interaction of classical and non-classical strains of *Bordetella* with *D. discoideum* to explore a potentially novel mode of transmission through the use of an environmental reservoir. In the second chapter of this thesis, we examine the role of key virulence factor genes in shedding, colonization, and transmission from infected mice to naïve mice.

Chapter 1: Classical and Non-Classical Bordetella species are capable of interacting with D. discoideum

Materials and Methods

The goal of the following experiments was to gain understanding of the strategies that bordetellae use to survive in the environment and transmit by examining the interactions of classical and non-classical *Bordetella* species with *D. discoideum*. Comparable genes between these species will be used to examine the genes responsible for avoiding predation by amoeba.

Inoculation and Growth of *D. discoideum*

Strains of bordetellae (Table 1) were grown in Luria Bertani (LB) broth until reaching mid-log phase, with an approximate concentration of $1x10^9$ CFU/ml. From these cultures, lawns were grown on standard SM5 media at 37°C for 48 hours. Lawns of *Klebsiella aerogenes* were also made on standard SM5 media, and acted as a control for the experiment. To grow *D. discoideum*, the bacterial lawns were inoculated with approximately $1x10^4$ PFU/mL spores previously collected from sori and incubated at 20°C for the length of the time course. Plaque growth of *D. discoideum* on the bacterial lawns was monitored at time points on days 4, 9, 16, and 23 post-inoculation. Plaque expansion of *D. discoideum* on the bacterial lawns was measured by marking plaque borders on plates at each of these time points. Areas of plaque expansion were photographed analyzed using ImageJ Software.

Bacterial Recovery of *Bordetella spp.* from *D. discoideum*

Starting on days 7-9 after the addition of amoeba to the lawns, and again one and two weeks after that, spores of *D. discoideum* were examined for the presence of *K. aerogenes* or *Bordetella* strains in sori. To do so, sori were picked using a micropipette tip, and resuspended in

PBS with a vortexer. *Bordetella* samples were diluted and plated on Bordet-Gengou agar (Difco) enriched with 10% defibrinated sheep's blood (Hema resources) and grown for 2 days at 37°C. *K. aerogenes* samples were diluted and plated on LB agar plates and grown for 1 day at 37°C.

D. discoideum **Spore-Count Assay**

To quantify the number of viable spores present in the sori of amoeba grown on *K. aerogenes* or bordetellae, sori were picked at weekly time points using a micropipette tip. The extracted sori were then re-suspended in PBS and vortexed. Re-suspended spores were combined at a 1:1 ratio with *K. aerogenes* grown in LB broth to mid-log phase ($1x10^9$ CFU/mL) at 37°C. Dilutions were plated on SM5 agar plates. After incubating for 72 hours at 20°C, plaques present on lawns of *K. aerogenes* were counted.

Results

Bordetellae species evade amoeba predation.

Amoebae such as *D. discoideum* are known to prey on bacteria. In the presence of a viable food source, such as *K. aerogenes, D. discoideum* will consume the bacterial lawn, forming a discernable plaque that grows over time (Figure 1). Recent data has shown that *B. bronchiseptica* is capable of resisting amoeba predation by *D. discoideum* (Bendor et al, 2016), and it is hypothesized that this trait is conserved amongst *Bordetella* species. Resisting amoeba predation is not seen in species such *K. aerogenes*, which are rapidly consumed by *D. discoideum* over time. For this reason, *K. aerogenes* acts as a control for this experiment.

Compared to *K. aerogenes, D. discoideum* growth appears limited on lawns of the tested *Bordetella spp.*, potentially suggesting that as a genus, bordetellae are capable of avoiding amoeba predation. In a time course comparing amoeba growth, the mean plaque growth on *K. aerogenes* (7607 mm^2 (n=8)) was 13 times that on *B.bronchiseptica* (584 mm^2 (n=8)). The plaques grown on *B. parapertussis* and *B. pseudohinzii* were similar phenotype to *B. bronchiseptica*, only expanding to an average of 930 mm^2 (n=5) and 197 mm^2 (n=8), respectively. In a time course comparing amoeba growth, plaques on *K. aerogenes* grew to 5881 mm^2 (n=3), almost 8 times the 784 mm^2 (n=4) of growth seen on *B. bronchiseptica*. Interestingly, *B. hinzii* strains differed in their phenotypes. *B. hinzii* strain *L60* allowed amoeba plaque growth similar to that seen with *B. bronchiseptica*, with an average plaque area of 484 mm^2 (n=6). Plaque growth on *B. hinzii* strain 5132 was significantly larger at an average of 2404

mm^2 (n=6). These results suggest that, like *B. bronchiseptica*, *B. pseudohinzii*, *B. parapertussis*, and *B. hinzii* strain L60 are capable of avoiding amoeba predation by *D. discoideum*. But it appears that *B. hinzii* strain 5132 does not. However standard deviation is high between samples. Overall, these data indicate a novel interaction of *Bordetellae* with amoeba, allowing species *B. bronchiseptica, B. parapertussis, B. pseudohinzii* and at least one strain of *B. hinzii* (L60) to survive consumption by *D. discoideum,* while typical bacteria such as *K. aerogenes* would fall victim to amoeba predation.

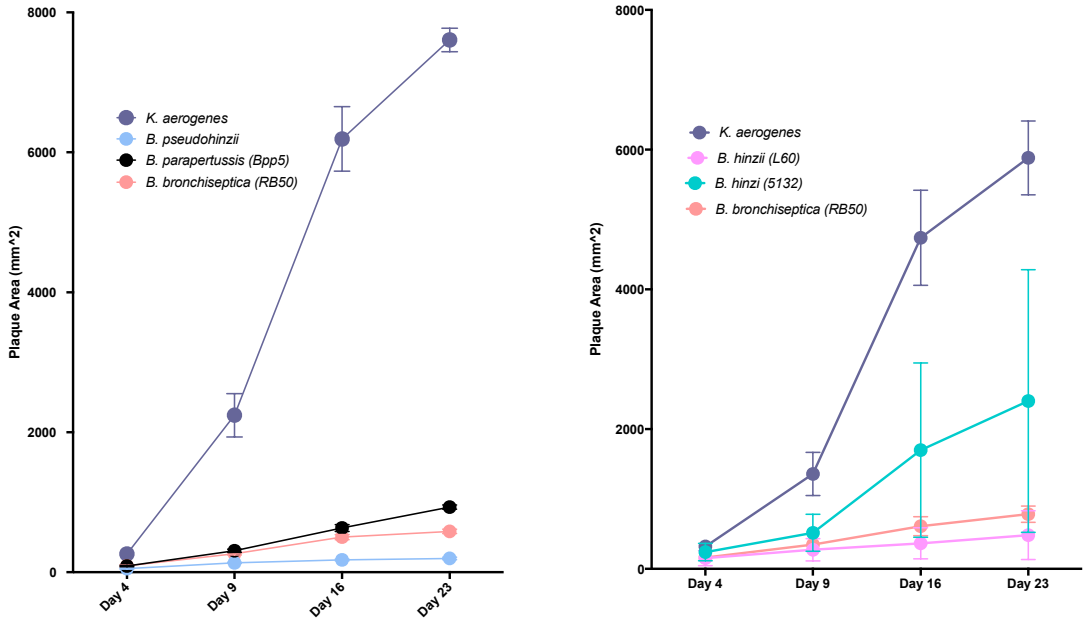

Figure 1. Classical and non-classical *Bordetella* spp. are capable of inhibiting plaque expansion of *D. discoideum* compared to *K. aerogenes*.

Lawns of indicated bacteria were inoculated with *D. discoideum*. At 4, 9, 16, 23 days post addition of amoeba, plaque expansion was measured. Error bars indicate SD.

Bordetella spp. survive within sori of *D. discoideum*

The inhibition of plaque growth by *Bordetellae* calls to question the reason behind this phenotype. Typical bacterial species utilized by amoeba, such as *K. aerogenes,* are consumed sorely as a food source. When sori were recovered from amoeba growing on plaques of *B. bronchiseptica,* an interesting observation was made. Despite the inhibiting of the growth of amoeba on *Bordetella spp,* classical and non-classical strains were all capable of localizing to the amoeba sori. (Figure 2) While *K. aerogenes* was incapable of localizing to the sori, *B. bronchiseptica* was recovered at high levels on days 9, 16, and 23. Likewise, *B. pseudohinzii*, *B. parapertussis*, and *B. hinzii* (both L60 and 5132 strains) were recovered at comparable levels from sori on days 9, 16, and 23. These findings were consistent throughout multiple time courses. These data suggest that *Bordetellae* can localize to the amoeba sori, using it to aid in transmission to disseminate throughout the environment and survive outside of a mammalian host.

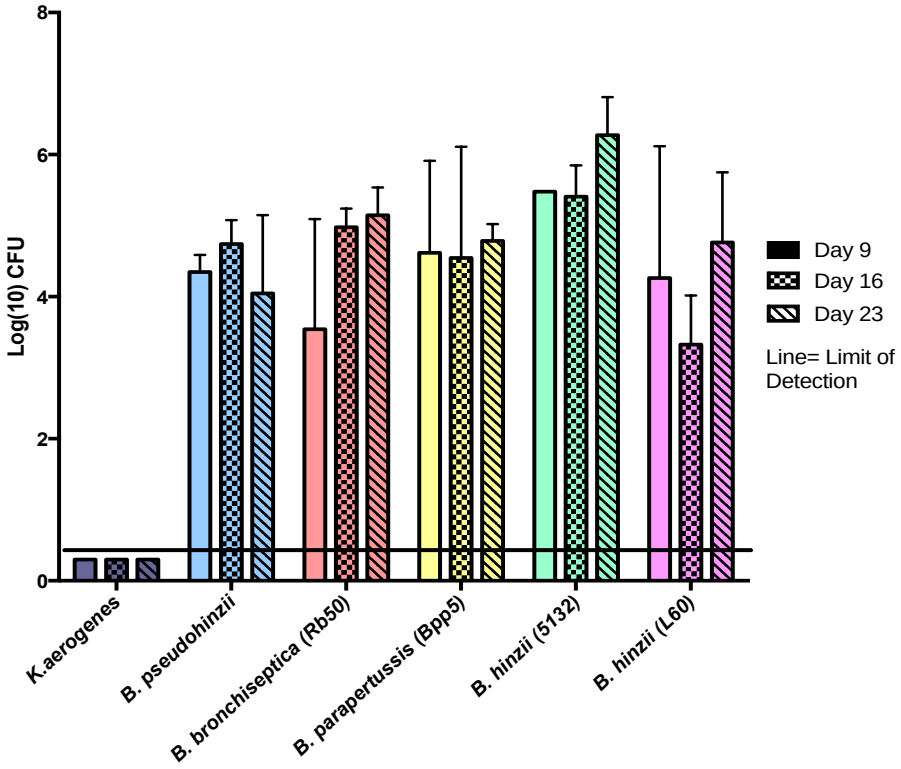

Figure 2. Classical and Non-Classical *Bordetella* Strains are capable of survival within sori of *D. discoideum*.

Bacterial recovery from *D. discoideum* sori grown on *K. aerogenes* (navy) and *Bordetella spp* (as indicated) on days 9, 16, and 23 post-addition of amoeba. Error bars indicate SD.

Bordetella spp.* do not prevent amoeba growth or lower spore counts of *D. discoideum

Due to the fact that bordetellae are capable of inhibiting plaque growth and surviving within the sori, it is hypothesized that this interaction may also affect the viability of spores within the sori of *D. discoideum*. Post addition of amoeba to lawns of *K. aerogenes* and *Bordetella spp.*, sori were picked, diluted, and plated to determine the viability of spores within the sori (Figure 3). Overall, it was found that spore counts within the sori of *D. discoideum* remained constant over the time and had no significant difference between *K. aerogenes* and *Bordetella* spp. Sori of amoeba grown on *K. aerogenes* showed consistent spore recovery numbers on days 9, 16, and 23, which was expected, as no bacteria was found within these sori.

Spore counts of sori picked from amoeba grown on *B. bronchiseptica* was highly consistent with *K. aerogenes*, varying little between days 9, 16, and 23. This was likewise for the other species, *B. pseudohinzii*, *B. parapertussis*, and *B. hinzii* (L60 & 5132). These data suggest that despite incorporation into the sori of *D. discoideum, Bordetella spp.* do not affect the viability of these amoeba. *Bordetellae* is shown here to co-exist within amoeba, strengthening the possibility of its use for environmental survival and transmission.

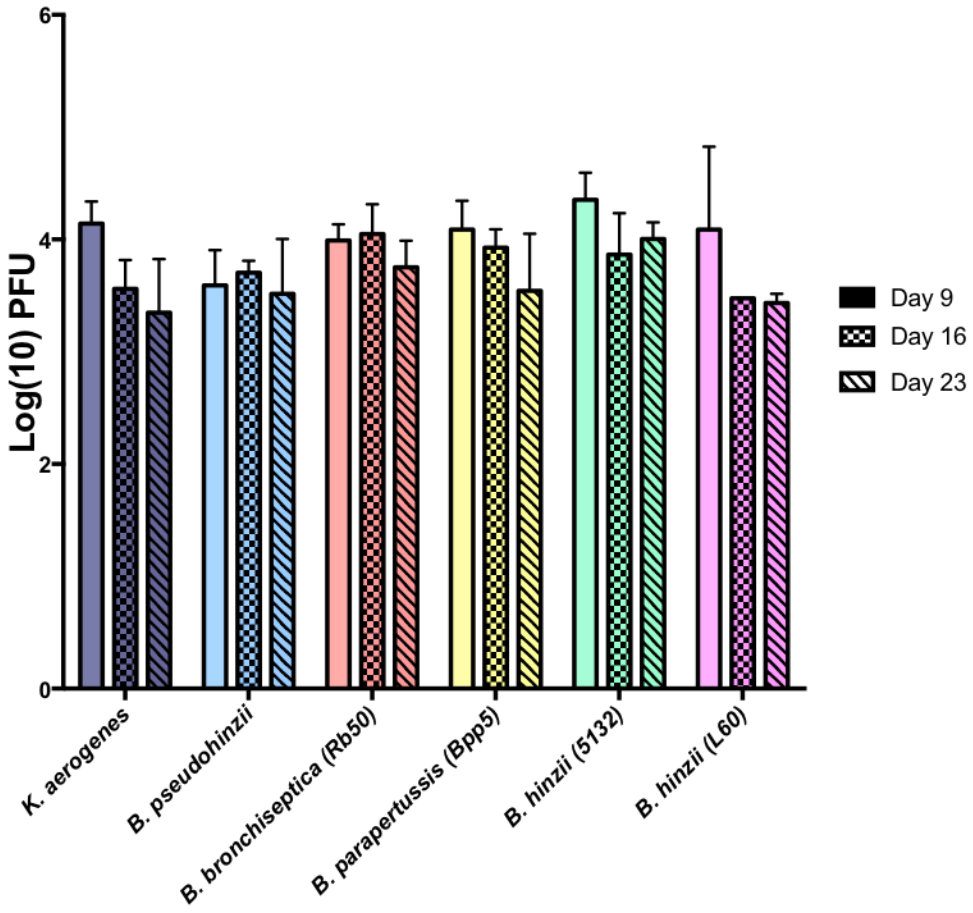

Figure 3. *D. discoideum* spores remain viable despite co-inhabitation with classical and non-classical *Bordetella* strains.

Spore recovery from *D. discoideum* sori grown on lawns of *K. aerogenes* (navy) and *Bordetella* strains (as indicated) on days 9, 16, and 23 post-addition of amoeba. Error Bars indicate SD.

Chapter 2: Using the Mouse Model to Study *Bordetella* spp. Transmission

Materials and Methods

The goal of this preliminary work was to identify target genes required for transmission of *Bordetella* spp. between mammalian hosts using an established mouse model. The results from this experiment can be used to identify potential vaccine targets for further testing in the more costly baboon model for *B. pertussis*. The proceeding outlines the experimental protocols to achieve these goals.

Institutional Care and Use Committee (IUCUC) Approval

This study is in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University at University Park, PA. (#46294 Bordetella-Host Interactions)

Experimental Protocol

This study used transgenic C3H/HeJ mice which express a defective form of Toll-Like Receptor-4, a critical component of the host's defense mechanism against *B. bronchiseptica* (Smallridge et al,. 2014). Mice were inoculated with 5 uL of a specific mutant strain of *B. bronchiseptica* (Table 1) at a concentration of 3×10^4 cfu/mL. For the initial mutant screening, mice were co-housed at a ratio of one inoculated mouse to two naïve mice. Beginning day 7 post-infection and continuing every other day, the external nares of infected and naïve individuals were swabbed to detect shedding of *B. bronchiseptica.* On day 21 post-infection, the mice were sacrificed. Target organs including the lungs, trachea, and nasal cavities of all infected

and naïve mice were homogenized then plated on Bordet Gengou (BG) agar supplemented with 10% defibrinated sheep's blood and 20 μg/mL streptomycin. Plates were incubated at 37°C for two days and then analyzed/enumerated for bacterial growth.

Table 1. Gene deletion mutants screened for transmission

Strain Tested	Gene Deletion	Function of Gene	Literature Cited
RB50	Wild-Type B. bronchiseptica	N/A	
RB53	Bvg+ phase-locked mutant	Virulence regulon is up-regulated	
RB50 Δ*bprl*	sRNA intergenic region		
RB50 Δ*bscN*	Component of Type III Secretion System	Necessary for colonization of the trachea.	Yuk, ca. 2000
RB50 Δ*dnt*	Dermonecrotic toxin	Deamidation of glutamine-63 Rho GTPases.	Schmidt et al., 1999
RB50 Δ*fimG*	Subunit of Type 1 Pili	Facilitates adhesion to host cells.	Busch and Waksman, 2012
RB50 Δ*prn*	Pertactin	Allows *Bordetella* to resist neutrophil-mediated clearance by host.	Inatsuka et al., 2010
RB50 Δ*pagL*	Lipid-A Modifying Enzyme	Biosynthesis of Lipid A, a component of LPS layer of cell wall.	MacArthur et al., 2011
RB50 Δ*arnt*	Lipid-A Modifying Enzyme	Addition of glucosamine to Lipid A, a component of LPS layer of cell wall.	Rolin et al., 2014
RB50 Δ*lpxO*	Lipid-Modifying Enzyme	Biosynthesis of Lipid A, a component of LPS layer of cell wall.	MacArthur et al., 2011
RB50 Δ*cap*	Component of Extracellular Polyaccharide Capsule (EPS)	Evasion of Host Innate Immunity, protection from complement mediated destruction	Parkhill et al., 2003

RB50 Δ*cap4*	"	"	Parkhill et al., 2003
RB50 Δ*cap9*	"	"	Parkhill et al., 2003
RB50 Δ*cap10*	"	"	Parkhill et al., 2003
RB50 Δ*cap11*	"	"	Parkhill et al., 2003

Results

Key virulence genes contribute to transmission amongst mice.

Successful transmission of *B. brochiseptica* requires the bacteria to accomplish three things; successful colonization (i.e. established infection in the inoculated host), shedding of the bacteria from the infected mouse, and transmission from infected mouse to naïve mouse. We hypothesize that key genes are required for transmission between hosts and that the loss of those genes will prevent transmission. Virulence factor mutants capable of colonization and shedding, but unable to transmit from infected to naïve hosts are of particular interest, as this inability suggests the significant of this virulence factor in transmission of *B. bronchiseptica*. Mutants were screened using a two-round system. If a mutant failed to transmit from one infected mouse to two naïve mice, the level of exposure was increased to two infected mice to two naïve mice and the mutant was tested again.

We tested a panel of virulence mutants for their ability to colonize and transmit. All the mutants tested were able to colonize the index infected mouse. (Table 2). During the first round of screening, mutants lacking *fhaA* or *bscN* were observed to transmit to the naïve mice. In contrast, RB53, and mutants lacking *bprL, fimG, dnt, lpxO, arnT, pagL, prn,* and capsule mutants failed to transmit. However, during the follow-up second round of the assay, transmission was observed for the RB53, *brpL*, and f*imG* mutants. Three mutants, those lacking *dnt, prn*, and the capsule, failed to transmit in this second, more rigorous test, confirming their role in transmission.

Table 2. Summary of mutant strains screened for transmission.

B. bronchiseptica mutant strains also tested for transmission. Round-2 Transmission entails increasing the number of inoculated mice from 1 to 2. N/A indicates that screening has not been completed.

Mutant	Colonization?	Round-1 Transmission?	Round-2 Transmission?
RB50 Δ*fha*	Yes	Yes	Yes
RB50 Δ*bscN*	Yes	Yes	Yes
RB50 Δ*bprl*	Yes	No	Yes
RB50 Δ*fimG*	Yes	No	Yes
RB50 Δ*dnt*	Yes	No	No
RB53	Yes	No	Yes
RB50 Δ*lpxO*	Yes	No	N/A
RB50 Δ*arnt*	Yes	No	N/A
RB50 Δ*pagL*	Yes	No	N/A
RB50 Δ*prn*	Yes	No	No
RB50 Δ*cap*	Yes	No	No
RB50 Δ*cap4*	Yes	No	No
RB50 Δ*cap9*	Yes	No	N/A
RB50 Δ*cap10*	Yes	Yes	N/A
RB50 Δ*cap11*	Yes	No	N/A

Failure to transmit suggests the capsule locus facilitates colonization and transmission

Of the mutants tested during this study, mutants deficient in the capsule locus (*Δcap*) of *Bordetella bronchiseptica* appeared most promising. The capsule locus of *B. bronchiseptica* encodes a layer of extracellular polysaccharide (EPS). In other bacterial species such as *Escherichia coli*, *Streptococcus spp.*, and *Bacillus anthracis*, capsules have been attributed to protection against complement-mediated immunity and evading the host immune system through the suppression of surface proteins (Dewan, 2016). On day 21-post inoculation, wild-type *B.*

bronchiseptica was recovered from nasal cavities of naive mice, indicating that transmission had occurred. For *B. bronchiseptica* mutants *Δcap, Δcap4, Δcap9, and Δcap11*, the lack of bacteria recovered from the nasal cavities of the naïve mice on day 21 showed a failure to transmit (Figure 4). However, one mutant on the capsule locus, *Δcap10,* was shown able to transmit, with bacterial levels recovered from the naïve mice significantly higher than wild-type *B. bronchiseptica.* These preliminary data indicate that the extra-polysaccharide capsule of *B. bronchiseptica* is necessary for transmission.

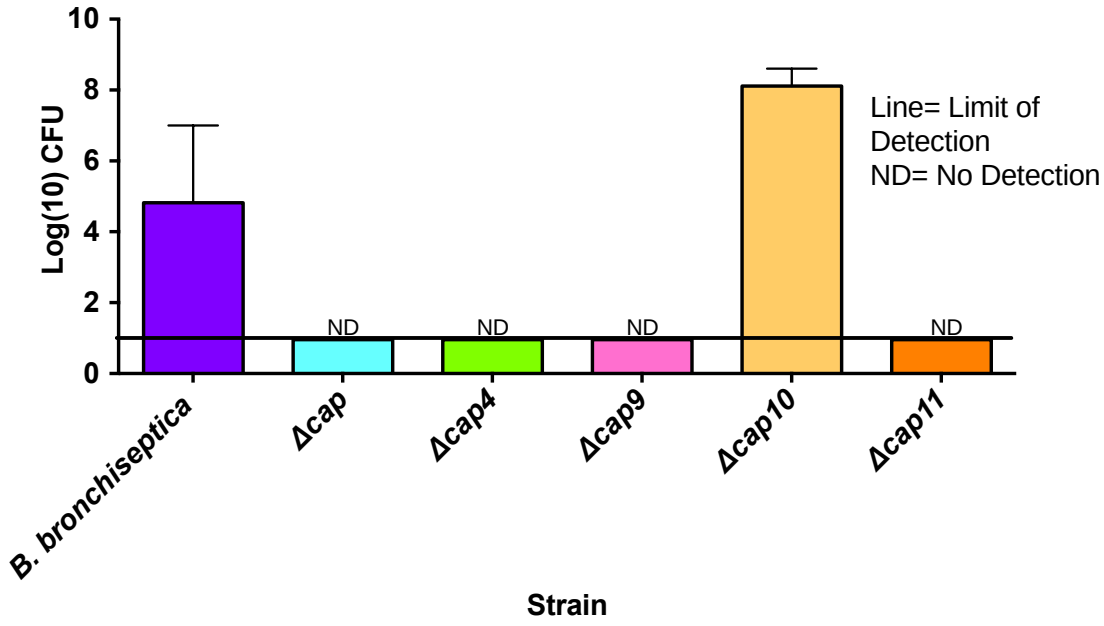

Figure 4. Transmission of *B. bronchiseptica* mutants from infected mice to naïve mice.

Graph depicts CFU of bacteria recovered from nasal cavities of naïve individuals during initial screen of *B. bronchiseptica* cap mutants. One infected mouse was co-housed with 2 naïve mice. Bacteria was recovered from nasal cavities of naïve mice on day 21 post-infection. Error indicates SD.

Encountering a mutant unable to transmit raises the possibility that transmission was not seen due to a failure to colonize in the inoculated mouse. However, as seen in figure 5, colonization was successful in all of the inoculated mice. The bacteria load (CFU counts) in the

nasal cavities of mice infected with *B. bronchiseptica* was high on day 21 post-infection. Similarly, mice inoculated with *Δcap, Δcap 4, Δcap9, Δcap10, and Δcap11* harbored comparable bacterial levels from the nasal cavity. This makes a failure to transmit due to a failure of exposure unlikely.

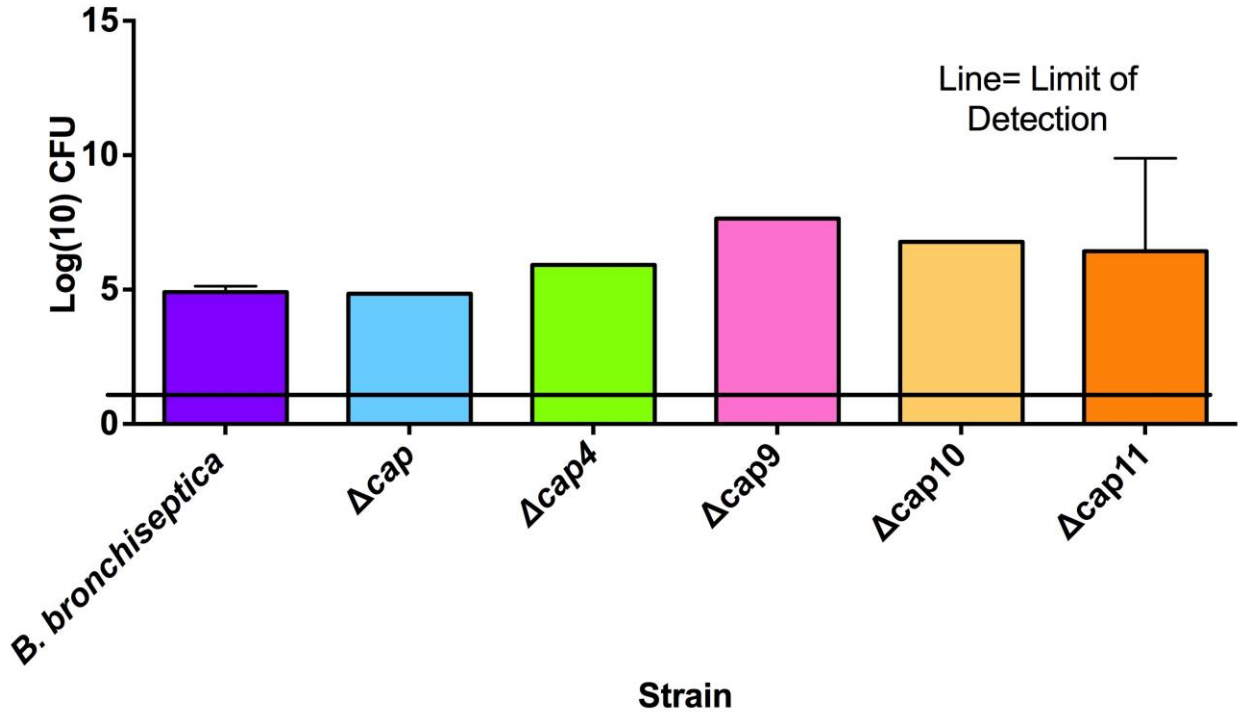

Figure 5. Colonization of *B. bronchiseptica* mutants in nasal cavities of infected mice.

Graph depicts CFU of bacteria recovered from nasal cavities of infected individuals during initial screen of *B. bronchiseptica* cap mutants. One infected mouse was co-housed with 2 naïve mice. Bacteria was recovered from nasal cavities of naïve mice on day 21 post-infection. Error indicates SD.

To rule out the question of failure to the capsule mutants failing to transmit due to insufficient exposure of naive mice to inoculated mice, a second screen was performed of the cap4 mutant. During this experiment, the level of exposure in the cage was increased, inoculating 2 mice instead of 1. In the case of *ΔfimG, Δbprl,* and *ΔbscN* mutants that were tested again

(Table 2), this was a sufficient increase to induce transmission. However, for *Δcap4*, an increase in exposure did not yield transmission to the naïve mice, despite high levels of bacterial recovery from the nasal cavities, trachea, and lungs of the inoculated mice (Figure 6). Therefore, these data support the idea that failure of *Δcap4* transmission was not due to insufficient exposure, and that genes missing from *Δcap4* mutant are involved in transmission of *B. bronchiseptica*.

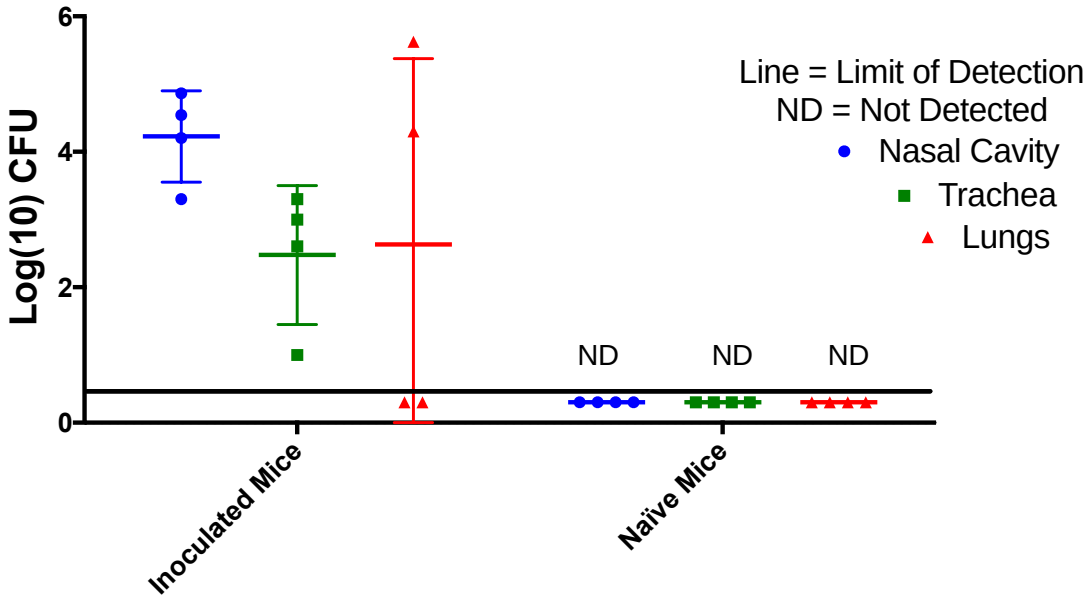

Figure 6. Round two screening of *B. bronchiseptica cap4* mutant.

Bacteria recovered from nasal cavities, trachea, and lungs of infected and naïve mice on day 21 post-infection. Error indicates SD.

Failure of B. bronchiseptica Δcap to transmit is attributed to a decrease in shedding over time

Since the ability of *B. bronchiseptica* to be transmitted amongst mice is dependent upon bacteria being shed from the nasal cavity and present on the external nares, we compared the amount of shedding between *B. bronchiseptica* and the capsule mutants. Shedding from *B. bronchiseptica* decreased slightly over time, but was persistently shed from the nares of infected

mice over the 20 days following inoculation. In contrast, mice infected with the capsule mutants showed a significantly lower and sporadic level of shedding from the nasal cavity compared to wild-type *B. bronchiseptica* (Figure 7). *Δcap* did not begin to shed until day 11 post inoculation, and maintained a significantly lower level of shedding than *B. bronchiseptica* for the rest of the experiment. Likewise, *Δcap4, Δcap9, Δcap10, and Δcap11* were significantly hindered in their ability to shed. Since both wild-type *B. bronchiseptica* and the capsule mutants possess the ability colonize and persist at similar levels in the nasal cavity of the mice, these results show that the distinction between the ability of *B. bronchiseptica* and capsule mutants to transmit likely lies in the inability to effectively shed from the infected host.

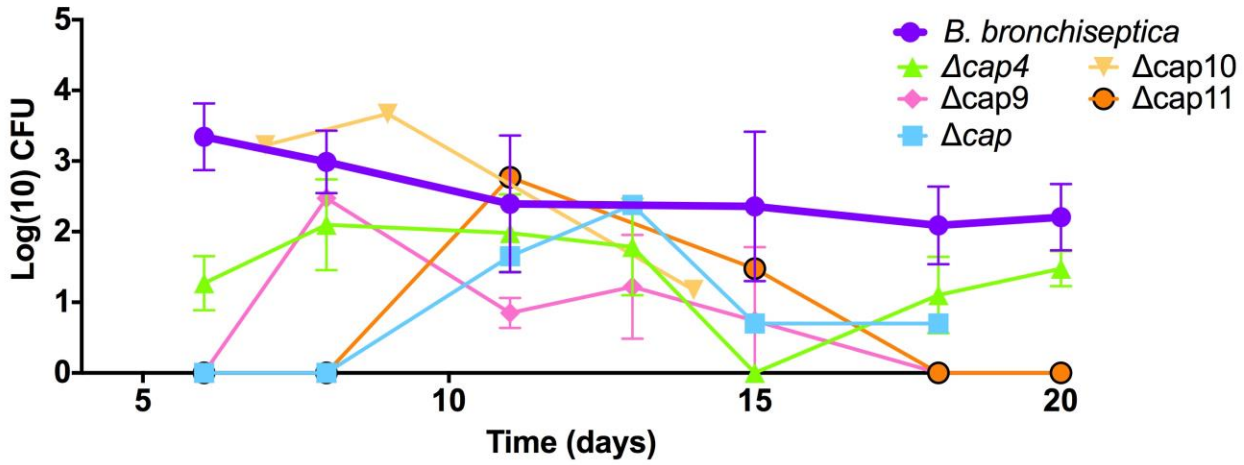

Figure 7. Shedding of B. *bronchiseptica* from infected mice

Bacteria recovered from nasal swabs of mice inoculated with wild-type and mutant *B. bronchiseptica* strains. Error indicates SD.

Discussion

Causing significant disease in human and veterinary medicine, infectious respiratory pathogens of *B. pertussis, B. bronchispetica, B. parapertussis*, among other species, are likely successful pathogens due to their ability to efficiently colonize, shed, and transmit from host to host. The recent increase in whooping cough (*B. pertussis*) incidence in humans has led to the increasing urgency to understand the mechanisms of transmission, much of which is still unknown. Previously, it has been thought that *Bordetella spp*. were only capable of spread through direct contact between infected individuals, through the spread of aerosol droplets. Herein, we describe the capability of multiple *Bordetella* species, *B. bronchiseptica*, B. parapertussis, *B. pseudohinzii*, and *B. hinzii* to utilize amoeba such as *D. discoideum* as a tool to survive outside of the host and disseminate throughout the environment. This idea presents an environmental niche of *Bordetellae* that has yet to be shown elsewhere.

More specifically, we first demonstrated that *B. bronchiseptica* is capable of avoiding predation of amoeba, while other bacterial species, particularly *K. aerogenes*, are incapable of this trait (Figure 1). Inhibiting bacterial consumption by the amoeba is necessary to persist and spread to new hosts. Furthermore, we recovered *B.bronchiseptica* from the sori of amoeba grown on lawns of *B. bronchiseptica*, showing that bordetellae can localize to the amoeba sori and remain viable (Figure 2). The highly complex life cycle of amoeba species such as *D. discoideum* includes a sori-bearing stalk as its final life stage, used to disseminate thousands of spores per sori to new locations plentiful in food. These data highly suggest that *Bordetella*

species can utilize the inherent disseminating ability of amoeba sori as a tool for environmental spread, and eventually transmission to a new host. Finally, despite surviving in the sori of amoeba, we showed that the presence of bordetellae in the fruiting body did not affect the amoeba spore counts within the sori (Figure 3). This suggests that *Bordetellae* do not harm the amoeba during this interaction, and this relationship is symbiotic.

Overall, the findings of the first chapter of this thesis highly suggest that *B. bronchiseptica* is capable of interacting symbiotically with amoeba such as *D. discoideum* and use it as an environmental reservoir to aid in transmission. Increasingly exciting, multiple species of *Bordetella* (*B. parapertussis, B. hinzii*, *B. pseudohinzii*) are capable of this interaction as well (Figure 2), suggesting that amoeba may play a role in the transmission cycles of the multiple species that bordetellae infect. These findings have significant implications, especially in the prevention of disease in livestock, who commonly come in contact with amoeba-bearing soils, water, and grass. Despite this, there is still more to examine before a definitive decision can be made regarding the role of amoeba in the transmission of *Bordetella* species. Perhaps one of the most significant species of *Bordetella* to human medicine, *B. pertussis,* has yet to be studied with *D. discoideum*.

Additionally, we have shown through the effective screening of numerous *B. bronchiseptica* mutants that there are multiple key virulence factors critical for transmission in vivo. This study presents an effective screen to study *B. bronchiseptica* transmission in murine hosts. Although understanding transmission between hosts is critical to controlling these highly infectious diseases, the study of bordetellae has previously focused on assays that measure the impact of "virulence factors" on aspects of colonization and pathogenesis within experimentally

inoculated animals, without regard for how they affect transmission between animals. To better understand the process of transmission between hosts, we developed effective conditions to study *B. bronchispetica* transmission between mice. Several mutants were shown to fail to transmit, while others were not, highlighting the capability of this model to screen for transmissibility of *B. bronchiseptica* mutants. The implications of this study are profound, as utilizing the mouse model is an efficient means by which to continue to identify genes important for *B. bronchiseptica* transmission, which are likely candidates for further study in the much more difficult and costly *B. pertussis* primate system.

Thus far from this model, we have discovered the importance of the capsule locus to *B. bronchiseptica* transmission. Herein, we have shown the extra-polysaccharide capsule of *B. bronchiseptica* is considered to have a major role in transmission, as *Δcap* mutants failed to transmit from infected mice to naïve mice (Figure 4). Furthermore, we showed that this failure to transmit was not due to colonization defect, as high numbers of bacterial were recovered from the nasal cavities of inoculated mice on day 21-post infection (Figure 5). The failure appears to be associated with a defect in shedding, due to the fact that all capsule mutants were significantly less effective at shedding from the nares of infected mice than wild-type *B. bronchiseptica* (Figure 7). From these data, it is shown that the capsule locus is necessary for efficient shedding and transmission of *B. bronchiseptica* in mice. However, a follow-up of the additional capsule mutants not screened in round-2 is necessary to make this conclusion, as *Δcap, Δcap9, Δcap10, and Δcap11* mutants were not tested to rule-out if insufficient exposure was the cause of transmission failure in these mutants.

In conclusion, this thesis aimed to investigate transmission dynamics of classical and non-classical *Bordetella* species, particularly *B. bronchiseptica,* to better understand how to disrupt transmission and prevent the spread of illness. In the first chapter of this thesis, we observed the interaction of classical and non-classical strains of *Bordetella* with *D. discoideum* to explore a potentially novel mode of transmission through the use of an environmental reservoir. Here it was found that *Bordetella* species *B. bronchiseptica, B. parapertussis, B. hinzii, and B. pseudohinzii* are capable of avoiding predation by *D. discoideum* and incorporating themselves into the sori of the amoeba, showing that the use of amoeba as an environmental reservoir is likely. In the second chapter of this thesis, we examined the role of key virulence factor genes in shedding, colonization, and transmission from infected mice to naïve mice, finding genes residing on the extra-polysaccharide capsule of *B. bronchiseptica* to be critical for shedding and transmission of the bacteria. From these data, we gain a better understanding of the environmental mechanisms behind transmission, as well as mechanisms behind some of the virulence factors involved in transmission.

References

Althouse BM, Scarpino SV. (2015). Asymptomatic transmission and the resurgence of *Bordetella pertussis*. *BMC Medicine*. 13, 146. doi:10.1186/s12916-015-0382-8.

Bendor, L. Taylor-Mulneix, DL. Linz, Bodo. Hilburger, LJ. Wagner, SM. Wilson, EF, Harvill, ET. (2016). The Bvg$^-$ phase facilitates growth and dissemination of *Bordetella bronchiseptica* along with the amoeba *Dictyostelium discoideum*. PLoS Biology. (Manuscript in Progress).

Bouchez, V., Brun, D., Cantinelli, T., Dore, G., Njamkepo, E., & Guiso, N. (2009). First report and detailed characterization of B. pertussis isolates not expressing pertussis toxin or pertactin. *Vaccine, 27*(43), 6034-6041. doi:10.1016/j.vaccine.2009.07.074

Busch, A., & Waksman, G. (2012). Chaperone-usher pathways: Diversity and pilus assembly mechanism. *Philosophical Transactions of the Royal Society B: Biological Sciences, 367*(1592), 1112-1122. doi:10.1098/rstb.2011.0206

Centers for Disease Control and Prevention. (2015). "Pertussis (Whooping Cough)." http://www.cdc.gov/pertussis/index.html.

Dewan, Kalyan K., Taylor-Mulneix, Dawn L., Hilburger, Lindsay. Preston, Andrew., & Harvill, Eric T. (2016) The *Bordetella bronchiseptica* Capsule Encoding Locus Facilitates Colonization and Transmission in a Mouse Model of Infection. (Manuscript in progress).

Eichinger, Louis, J. A. Pachebat, G. Glöckner, V. A. Rajandream, R. Sucgang, M. Berriman, J. Song, R. Olsen, K. Szafranski, Q. Xu, B. Tunggal, and A. Kuspa. "The Genome of the Social Amoeba Dictyostelium Discoideum." *Nature* 435 (2005): 43-57. Web.

Finger, H., & Wirsing von Koenig, C. H. (1996). Chapter 31: Bordetella. In S. Baron (Ed.), *Medical Microbiology* (4th ed.). Galveston, TX: University of Texas Medical Branch at Galveston.

Inatsuka, C. S., Xu, Q., Vujkovic-Cvijin, I., Wong, S., Stibitz, S., Miller, J. F., & Cotter, P. A. (2010). Pertactin Is Required for Bordetella Species To Resist Neutrophil-Mediated Clearance. *Infection and Immunity, 78*(7), 2901-2909. doi:10.1128/iai.00188-10

Lam, C., Octavia, S., Ricafort, L., Sintchenko, V., Gilbert, G. L., Wood, N., . . . Lan, R. (2014). Rapid Increase in Pertactin-deficient Bordetella pertussis Isolates, Australia. *Emerging Infectious Diseases, 20*(4), 626-633. doi:10.3201/eid2004.131478

Lamberti, Y. A., Hayes, J. A., Vidakovics, M. L., Harvill, E. T., & Rodriguez, M. E. (2010). Intracellular Trafficking of Bordetella pertussis in Human Macrophages. *Infection and Immunity, 78*(3), 907-913. doi:10.1128/iai.01031-09

Macarthur, I., Jones, J. W., Goodlett, D. R., Ernst, R. K., & Preston, A. (2011). Role of pagL and lpxO in Bordetella bronchiseptica Lipid A Biosynthesis. *Journal of Bacteriology, 193*(18), 4726-4735. doi:10.1128/jb.01502-10

Nicholson, T. L., Brockmeier, S. L., Loving, C. L., Register, K. B., Kehrli, M. E., Stibitz, S. E., & Shore, S. M. (2011). Phenotypic Modulation of the Virulent Bvg Phase Is Not Required for Pathogenesis and Transmission of Bordetella bronchiseptica in Swine. *Infection and Immunity, 80*(3), 1025-1036. doi:10.1128/iai.06016-11

Otsuka, N., Han, H., Toyoizumi-Ajisaka, H., Nakamura, Y., Arakawa, Y., Shibayama, K., & Kamachi, K. (2012). Prevalence and Genetic Characterization of Pertactin-Deficient Bordetella pertussis in Japan. *PLoS ONE, 7*(2), E31985. doi:10.1371/journal.pone.0031985

Paddock, C. D., Sanden, G. N., Cherry, J. D., Gal, A. A., Langston, C., Tatti, K. M., . . . Zaki, S. R. (2008). Pathology and Pathogenesis of Fatal Bordetella pertussis Infection in Infants. *Clinical Infectious Diseases, 47*(3), 328-338.

Parkhill, Julian, Mohammed Sebaihia, Andrew Preston, Lee D. Murphy, Nicholas Thomson, David E. Harris, Matthew TG Holden, Carol M. Chrucher, Stephen D. Bentley, Karen L. Mungall,… Duncan J. Maskell. "Comparative Analysis of the Genome Sequences of Bordetella Pertussis, Bordetella Parapertussis and Bordetella Bronchiseptica." *Nature Genetics* 35 (August 10, 2003): 32-40. doi:doi:10.1038/ng1227.

Rolin, O., Muse, S. J., Safi, C., Elahi, S., Gerdts, V., Hittle, L. E., . . . Pirofski, L. (2013). Enzymatic Modification of Lipid A by ArnT Protects Bordetella bronchiseptica against Cationic Peptides and Is Required for Transmission. *Infection and Immunity, 82*(2), 491-499. doi:10.1128/iai.01260-12

Schmidt, G., Goehring, U., Schirmer, J., Lerm, M., & Aktories, K. (1999). Identification of the C-terminal Part of BordetellaDermonecrotic Toxin as a Transglutaminase for Rho GTPases. *Journal of Biological Chemistry*, *274*(45), 31875-31881. doi:10.1074/jbc.274.45.31875

Smallridge, W., et al. (2014). Understanding How Vaccination and Particular Virulence Factors Contribute to Bordetella Transmission. Graduate Thesis, The Pennsylvania State University. Department of Immunology and Infectious Diseases. University Park, PA 16802

Swanson A. R., Vadell E. M., Cavender J. C. (1999). Global distribution of forest soil dictyostelids. *J. Biogeogr*. 26, 133-148.

Warfel, J. M., Beren, J., Kelly, V. K., Lee, G., & Merkel, T. J. (2012). Nonhuman Primate Model of Pertussis. *Infection and Immunity*, *80*(4), 1530-1536. doi:10.1128/iai.06310-11

Lindsay J. Hilburger
Ljh5263@psu.edu

EDUCATION

Bachelor of Science

Veterinary and Biomedical Sciences — Anticipated December 2016

The Pennsylvania State University. University Park, PA

Minor: Equine Science

- Schreyer Honors Scholar
  - Thesis Title: A study of the genes required for Bordetella Transmission and Bordetella's Interaction with Dictyostelium discoideum.
  - Honors Advisor: Lester Griel

WORK EXPERIENCE

Summer Research Fellowship — May-August 2016

San Diego Zoo Institute for Conservation Research

Escondido, CA 29020

- Pioneered a research project studying disease outbreak in the zoo's flock of Rainbow Lorikeets.
- Worked alongside pathology department to perform necropsies of deceased collection animals.

Volunteer — March 5-11, 2016

World Vets

Granada, Nicaragua

- Volunteered with organization to provide free veterinary care to local communities.

Undergraduate Researcher — Jan 2015-Present

Eric T. Harvill Lab
Center for Infectious Disease Dynamics
Penn State University. University Park, PA

Internship May 2014-June 2014
Daniel Kriel, DVM
Limpopo, South Africa

◦ Participated in wildlife game capture. Tasks included administering injections, drawing-up medications, blindfolding animals, assisting in lifting and relocating animals, and shadowing veterinarian.

Internship June 2014-August 2014
Rick Parsons, DVM
Churchville Veterinary Clinic
Churchville, NY 14422

◦ Assisted veterinarian by restraining animals, gathering supplies for procedures, cleaning procedure rooms, and caring for hospital residents.
◦ Performed diagnostic lab work and interpreted results. (stool, urine, blood, ear swabs)

TEACHING EXPERIENCE

Teaching Assistant- Animal Science 201 Spring 2016-Present
◦ Lead a laboratory section of approximately 20 students.
◦ Responsible for creating and grading lab quizzes and assignments.

HONORS AND AWARDS

Spring 2016 Undergraduate Research Grant (Penn State): Using a mouse model to determine the genes involved in Bordetella transmission.

◦ Grant awarded to students pursuing individual research projects. $2000

Summer 2015 Undergraduate Research Grant (Penn State): Determining the ability of Bordetella species to avoid predation by Dictyostellium discoideum during growth and transmission.

◦ Grant awarded to students pursuing individual research projects. $3000

NESA (Northeast Student Affiliate)- 1st Place Independent Research Presentation

Presentation Title: Bordetella Transmission Amongst Mice.

POSTER PRESENTATIONS

Poster Title: Bordetella Transmission amongst mice

Presented at:

◦ International Bordetella Symposium

Buenos Aires, Argentina. April 5-8 2016

◦ Gamma Sigma Delta Research Expo

Penn State University. March 28, 2016

Poster Title: Birds of a Feather Get Sick Together? A Disease Investigation of Rainbow Lorikeets

Presented at:

◦ San Diego Zoo Institute for Conservation Research