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IMMUNE-MEDIATED EVASION OF THE ADAPTIVE IMMUNE RESPONSE BY THE BORDETELLAE

A Dissertation in

Immunology and Infectious Diseases

by

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ABSTRACT

The *Bordetella*, a genus composed of nine species of gram negative bacteria, include *B. bronchiseptica, B. pertussis,* and *B. parapertussis*, three mammalian respiratory pathogens. *B. bronchiseptica* is frequently isolated from a wide range of non-human mammals, causing respiratory diseases such as kennel cough and atrophic rhinitis. *B. pertussis* and *B. parapertussis* are the causative agents of whooping cough, a disease that affects an estimated 50 million people annually worldwide. Interestingly, both *B. pertussis* and *B. parapertussis* evolved independently from a *B. bronchiseptica*-like progenitor, but do not induce reciprocal immunity due to the expression of O-antigen by *B. parapertussis*. Here we show that while *B. bronchiseptica* is susceptible to *B. pertussis* – induced immunity, *B. parapertussis* is able to avoid this immunity by requiring an antibody - mediated immune response against O-antigen for protection. In addition to being a protective antigen, O-antigen also facilitates *B. parapertussis* colonization of the respiratory tract via inhibiting complement component C3 binding and subsequent lysis. Together, these data offer an explanation for the apparent host tropism of *B. bronchiseptica* to non-human mammals and the circulation of two closely related pathogens *B. pertussis* and *B. parapertussis* in human populations.

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LIST OF ABBREVIATIONS

aP: Adacel, acellular Pertussis vaccine
BG: Bordet-Gengou
CFU: Colony Forming Units
CR3: Complement Receptor Type III
CVF: Cobra Venom Factor
HRP: Horseradish Peroxidase
IFN: Interferon
IL: Interleukin
i.p.: Intraperitoneal
LPS: Lipopolysaccharide
mAb: Monoclonal Antibody
Mbp: Megabasepairs
PBS: Phosphate Balanced Saline
TLR: Toll-Like Receptor
TGF: Tumor Growth Factor
TNF: Tumor Necrosis Factor

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Chapter 1: Introduction

The Genus *Bordetella*: The genus *Bordetella,* containing nine species; *B. pertussis, B. parapertussis*$_{hu}$*, B. parapertussis*$_{ov}$ (ovine-adapted)*, B. bronchiseptica, B. avium, B. hinzii, B. holmesii*, *B. trematum* and *B. petrii*, are gram negative pleomorphic rods, a subset of which are important mammalian pathogens (55). *B. parapertussis*$_{ov}$ is not studied in this manuscript; therefore all subsequent references to *B. parapertussis* will indicate the human-adapted *B. parapertussis*. The classical bordetellae, *Bordetella pertussis, B. parapertussis,* and *B. bronchiseptica*, are closely related respiratory pathogens that infect mammals. *B. bronchiseptica* infects a wide range of mammals, including dogs, rabbits, pigs, mice, cats, horses, cows, seals, guinea pigs, and monkeys, causing anything from asymptomatic infections to lethal pneumonia (28, 55). *B. pertussis* and *B. parapertussis* cause the classic childhood illness, whooping cough (80). This disease is characterized by paroxysmal coughing fits followed by the namesake whoop as air is inhaled though mucus clogged airways. The severe coughing can last for 3 months and causes death in approximately 1 in every 150 infants infected with these pathogens (11, 55). It is estimated that 50 million cases of whooping cough occur worldwide each year, although as few as 1 in 600 *B. pertussis* or *B. parapertussis* infections may be reported (20, 25). Commercially available *B. pertussis* vaccines offer transient protection lasting only five to ten years (35, 55). While these vaccines effectively prevent *B. pertussis*-caused disease for a limited time, the vaccines are derived solely from *B. pertussis* components and provide little if any protection against *B. parapertussis* infection (24, 82).

Evolution of *B. pertussis* and *B. parapertussis*: *B. bronchiseptica* has the largest genome among the classical bordetellae, containing approximately 5.3 Mbp (61). This genome includes almost all the genes found in both *B. pertussis* and *B. parapertussis*, sized approximately 4.0 Mbp and 4.7 Mbp respectively (61). Genomic comparision suggests that a *B. bronchiseptica*-like progenitor gave rise to both human adapted bordetellae and the evolution of *B. pertussis* and *B. parapertussis* has been accompanied by a large-scale loss of genes, such as genes responsible for O-Antigen biosynthesis in *B. pertussis* (22, 31, 57, 66). Therefore, *B. bronchiseptica* likely also contains all the necessary genes for human infection. Microarray and Western Blot analyses have shown that *B. bronchiseptica* also expresses a majority of the genes known to be virulence factors for all three pathogens (22, 28, 61). This is interesting as *B. bronchiseptica* is extremely successful at infecting and persisting in a diverse host range including monkeys, but is rarely found in humans (12, 55).

***Bordetella* Virulence Factors:** The bordetellae have an impressive arsenal of virulence factors which allow these bacteria to colonize, persist and avoid host immunity while surviving in the respiratory tract. Pertactin and filamentous hemagglutinin aid in adherence to epithelial cells and isogenic mutants lacking these factors show

decreased colonization in the lower respiratory tract (21, 29). All three *Bordetella* species secrete adenylate cyclase toxin that intercolates into the membrane of host cells and causes intoxication by increasing cAMP levels (5, 36). This toxin also gives the *Bordetella* the characteristic β-hemolysis seen on Bordet-Gengou agar plates. The potent virulence factor pertussis toxin is only expressed by *B. pertussis*, and inhibits neutrophil migration into the lungs, allowing *B. pertussis* to avoid clearance from the lungs (40, 55). Both *B. bronchiseptica* and *B. parapertussis* contain the pertussis toxin gene, but do not express this operon due to mutations in the promoter region (59, 60).

The Lipopolysaccharide (LPS) structure also varies within the *Bordetella* genus. LPS consists of 3 major components (78). The lipid A region anchors the LPS to the bacterial cell, and is responsible for the Toll-like Receptor (TLR)-4 signaling induced by this factor (58). The core oligosaccharide region can be heavily modified with branching saccharide moieties, adds structure and support for the LPS molecule, and serves to connect the lipid A region to the outer-most region of LPS, the O-Antigen (78). O-Antigen, a repeating chain of disaccharides, is the most variable portion of LPS and is largely responsible for bacterial serotypes (16). O-Antigen has also been implicated in serum resistance, proper expression and/or function of extracellular factors and virulence *in vivo* (3, 8, 14, 16, 32, 48, 62, 70, 72-74, 78, 84). *B. bronchiseptica* and *B. pertussis* have identical core oligosaccharides (69) with heavy modifications of the lipid A and trisaccharide regions that are mediated, at least in part, by the *pagP* and *wlb* loci, respectively (1, 68). *B. parapertussis* has a truncated core oligosaccharide that lacks the trisaccharide modification (66). Interestingly, *B. parapertussis* contains the *wlb* locus which encodes the required genes, indicating that the lack of modification is not due to the absence of the biosynthetic pathway (69). One of the most interesting differences in the LPS structures among the *Bordetella* is the O-Antigen serotype. *B. pertussis* completely lacks O-Antigen due to a natural 20kb deletion in the *wbm* locus (61, 67). *B. bronchiseptica* and *B. parapertussis* both express an O-Antigen, however, in *B. parapertussis*, seven of the *wbm* genes are unique to the classical *Bordetella* (66), causing the O-Antigen expressed by *B. parapertussis* to be antigenically distinct from the prototypical *B. bronchiseptica* O-Antigen.

The Immune System: The immune system has two major components responsible for the control and clearance of pathogens from the host. The innate immune system is the first line of defense and has multiple functions. The first barrier that a pathogen must breach is the epithelial layer of cells in the skin or mucosal tissues such as the lungs and gastrointestinal tract. If a pathogen successfully penetrates the epithelial layer, a protein cascade called complement will activate (33, 76). Complement proteins are heat-labile proteins that bind to the surface of the pathogen through three different mechanisms, the classical pathway, the alternative pathway and the mannose-lectin binding pathway (30, 33). The classical pathway complement components C1, C4, and

C2 are activated by antibody-binding to the surface of the pathogen. The alternative pathway is initiated by the direct binding of complement component C3 followed by Factor B and D to the pathogen and does not required antibodies (33). The mannose-lectin pathway deposits MASP, C4 and C2 on the peptidylglycan found in some bacterial pathogens (30). All three pathways converge on C3 which causes four major effects; it amplifies the cascade, releases a chemoattractant C3a, produces the ligand for complement receptor C3 (CR3), and allows for C5 binding. C5, C6, C7, C8 and C9 all form the Membrane Attack Complex which punches a hole in the membrane and can cause the lysis of the pathogen(30, 33).

If the complement cascade is not sufficient to eliminate the pathogen, phagocytic cells such as neutrophils, macrophage and dendritic cells will migrate to the area of infection with the aid of pro-inflammatory chemokines and cytokines such as Tumor Necrosis Factor (TNF)-α, C3a, C5a, Interleukin (IL)-1, and IL-8 (33, 76). These cells express CR3, scavenger receptors, TLR and antibody (Fc) receptors and can endocytose, degrade and present the broken down pathogen to the second portion of the immune system, the adaptive immune system (76, 79).

The adaptive immune system is the flexible, ever-changing branch of the immune system which can specifically recognize and develop a memory response to every pathogen that is encountered throughout the lifespan of the host. B cells mature in the bone marrow and go through both positive and negative selection to ensure the production of functional αβ or γδ chains (15, 19). These cells produce antibodies which can act as flags for complement binding and phagocytosis, aiding in pathogen removal. Antibodies also can neutralize secreted toxins, opsinize foreign materials and cause agglutination (15). T cells mature in the thymus, in a strictly regulated environment with both positive and negative selection to prevent self-recognition (46, 81). Two major T cell types, CD4+ and CD8+, had distinct functions. CD4+ T cells produce cytokines such as Interferon (IFN)-γ, IL-4, IL-10, IL-12, and IL-17, and TGF (Tumor Growth Factor)-β that aid in antibody production, neutrophil and macrophage activation and, eventually, down-regulation of the adaptive immune response after the clearance of the pathogen (15, 46, 76, 81). CD8+ T cells are cytotoxic cells that kill cancerous cells and virus-infected cells. Both T and B cells are able to go quiescent for many years and reactivate upon exposure to the pathogen, allowing the host to rapidly respond to that pathogen later in life (81).

Murine Model of Infection: The murine model of infection has several advantages to other commonly studies models such as yeast, *Drosophila*, nematodes, and zebrafish. While these less complex model organisms are conducive to studying cell signaling, development and innate immune responses, the mouse model more closely mimics humans in the nervous, endocrine, skeletal-muscular and adaptive immune systems (75). Specific to the *Bordetella*, infected mice share many disease characteristics with

infected humans, such as the production of *Bordetella*-specific antibodies, acute lymphocytosis, neutrophil migration to the lungs and a memory T and B cell response (55). The mouse model also offers genetically identical hosts lacking specific genes involved in the immune system, can be rapidly bred, are cost-effective to maintain, and can easily be infected with the classical *Bordetella* (38, 41, 75). However, the mouse model is not perfect, and differences between humans and mice exist.

The most notable difference between the clinical signs of *Bordetella* infection in mice and humans is the inability of mice to cough. This lack of coughing may also explain the lack of transmission of *Bordetella* between mice in a laboratory setting. Other, more subtle differences also exist. *Bordetella* infection in humans is generally accompanied by a rise in body temperature (55), while several studies have shown that mice will drop in body temperature due to a respiratory infection (42). Substantial differences exist between IgA receptors expressed by mice and humans (26). IgA contributes to the control of *B. bronchiseptica* in the nasal cavity of mice, but did not affect *B. pertussis* or *B. parapertussis* colonization in the murine model (85). However, transgenic mice expressing the human FcαRI, to which there is no homolog in mice, were more efficient at clearing *B. pertussis* from the respiratory tract (39), suggesting that additional IgA-mediated control may allow for more efficient protection against the *Bordetella* in humans. The difference in IgA-mediated immunity may also contribute to the persistence of *B. parapertussis* in the nasal cavity of mice, but not in humans (41, 85). While *B. parapertussis* has not yet been examined the transgenic FcαRI mice, it would be interesting to determine if this receptor allowed for the clearance of *B. parapertussis* from the murine nasal cavity. Despite these differences, the mouse model offers the best in vivo infection model available for the dissection of the host response to *Bordetella* infections.

Infection Kinetics and Host Immunity: A normal course of *B. pertussis* infection in wild type mice peaks at day 7 post-inoculation with approximately 10^7 CFU distributed throughout the respiratory tract. The CFU count steadily declines over 3 weeks until clearance, seen about day 28 to day 49 post-inoculation (37, 41). *B. bronchiseptica* and *B. parapertussis* infections follow a similar course in the lower respiratory tract, however these bacteria persist in the nasal cavity for the life of the animal (41).

Several host immune factors are required for the control and clearance of a primary *Bordetella* infection. *B. bronchiseptica* infection is lethal to TLR4-deficient mice within the first 3 days. TLR4 signaling in response to *B. bronchiseptica* leads to a robust TNF - α response, which is also required for survival of the first 3 days of infection (52-54). *B. pertussis* induces less of a TLR4-mediated TNF-α response and TNF- α knockout mice infected with *B. pertussis* show a late requirement for TNF- α, dying approximately 2 weeks post-inoculation (87). Interestingly, survival of a *B. parapertussis* infection does not require either TLR4 or TNF-α [unpublished data].

Complement is also important in the control of *B. bronchiseptica* infection as CD11b knockout mice which lack Complement 3 Receptor succumb to the infection between days 3 and 4 post-inoculation (63). Fcγ Receptors have also been shown to aid in the clearance of the *Bordetella* from the respiratory tract (65). Mice that lack either B cells or T cells never clear the bordetellae from the lower respiratory tract (41, 44, 86). Mice that lack both B and T cells succumb to *B. bronchiseptica* and *B. parapertussis* infections between day 45-60 or day 17-21 post-inoculation, respectively (13, 86). IFN -γ knockout mice have decreased neutrophil recruitment and exhibit delayed clearance of all three classical *Bordetella* (4, 44, 56, 64). Therefore, several innate immune factors and the adaptive immune response are required to control and clear *Bordetella* infections.

Incidence of *Bordetella* Disease: *B. bronchiseptica* is extremely prevalent in companion and agricultural mammals, with greater than 90% seroprevalence in swine herds, 75% culture-positive in rabbits, and 11% culture-positive in felines from a variety of sources (10, 27). However, *B. bronchiseptica* has not been associated with a disease epidemic in immunocompetent humans. It is thought that host limitations of many pathogens may be due to receptor/ligand specificity between the pathogen and host (6, 17, 43, 49, 77). However, individual *B. bronchiseptica* strains have been shown to efficiently infect a wide range of mammals (unpublished data). Furthermore, *B. bronchiseptica* strains from across the phylogenetic tree have been isolated from humans (2, 7, 28, 34, 47, 88, 89), suggesting that *B. bronchiseptica* strains are not limited to non-human mammals. The rarity of *B. bronchiseptica* isolation from humans could be explained by basic ecological theories indicating that two closely related immunizing pathogens should not co-exist in the same host populations, as cross immunity will lead to immune-mediated competition (17, 31, 49, 71). Indeed, over 90% of humans in a population are seropositive for *B. pertussis*, indicating immunity to this pathogen is widespread (25). Therefore, immune-mediated competition conferred by *B. pertussis* may limit the circulation of *B. bronchiseptica* in human populations.

Interestingly, *B. parapertussis* is routinely isolated from human populations in which *B. pertussis* is endemic (9, 25, 35), and our lab has recently shown that this pathogen is able to avoid *B. pertussis*-induced immunity (84). However, the actual incidence of *B. parapertussis* is difficult to determine due to several factors. *B. parapertussis*-caused disease is clinically indistinguishable from *B. pertussis*-caused disease, although retrospective studies have indicated that *B. parapertussis* cases may be, on average, of shorter duration (9, 35). In addition, differential diagnosis does not facilitate treatment, and therefore is not routinely performed in the clinical setting. To further compound the problem, the Center for Disease Control has labeled *B. parapertussis* as a non-reportable agent (18). Studies specifically aimed at determining the incidence of *B. parapertussis* verses *B. pertussis* cases has seen considerable

variation, with the percentage of whooping cough cases caused by *B. parapertussis* ranging from ~1% to ~98% in a given outbreak (83). Seroprevalence data suggests that ~60% of a population is seropositive for *B. parapertussis* (50, 51), however, it is unknown when or if immunity against this pathogen wanes (25). *B. parapertussis* causes a higher percent of whooping cough cases in vaccinated, compared to unvaccinated, individuals (45). In addition, the resurgence of whooping cough roughly correlated to the transition to the use of acellular pertussis vaccines (18, 45) which offer no protection against *B. parapertussis*, and may actually enhance the ability of *B. parapertussis* to colonize hosts (23).

Preview: This dissertation will examine the effects of *B. pertussis*-induced immunity on both *B. bronchiseptica* and *B. parapertussis* challenge in the murine model. In synopsis, *B. bronchiseptica* is sensitive to both *B. pertussis*-infection and -vaccination induced immunity due to shared antigens pertactin and filamentous hemagglutinin. Vaccination with these purified *B. pertussis* proteins or a current pertussis acellular vaccine containing these proteins also decreased *B. bronchiseptica* colonization in the lower respiratory tract. *B. parapertussis* also shares these antigens, but is able to avoid *B. pertussis*-induced immunity. *B. parapertussis* expresses an O-antigen which prevents *B. pertussis*-specific antibodies from binding to the surface of the bacteria. This lack of binding of *B. pertussis*-induced antibodies may contribute to the ability of *B. parapertussis* to circulate in *B. pertussis*-immune populations (84). An immune response to the O-antigen of *B. parapertussis* is important in controlling *B. parapertussis* challenges. Infection or vaccination with an isogenic mutant of *B. parapertussis* that is unable to produce an O-antigen induces less protection upon secondary challenge and significantly less *B. parapertussis*-specific antibodies. In addition, passive transfer of antibodies raised against *B. parapertussis* lacking O-antigen is ineffective at reducing *B. parapertussis* colonization. Therefore, O-antigen is a critical protective antigen for *B. parapertussis*, and may be considered as a candidate for inclusion in current whooping cough vaccines. O-antigen also serves to protect *B. parapertussis* from complement component C3 deposition and C5-mediated killing. In addition, O-antigen is required for this bacterium to systemically spread in immunodeficient mice, a requirement that is abrogated upon the depletion of complement. In conclusion, Immune-mediated competition among the bordetellae may explain the apparent host tropism of *B. bronchiseptica* to non-human mammals and how *B. parapertussis* is able to avoid the same selective pressures and persist in largely *B. pertussis*-immune populations.

References:

1. **Allen, A., and D. Maskell.** 1996. The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in Bordetella pertussis. Mol Microbiol **19:**37-52.
2. **Amador, C., E. Chiner, J. L. Calpe, V. Ortiz de la Table, C. Martinez, and F. Pasquau.** 1991. Pneumonia due to Bordetella bronchiseptica in a patient with AIDS. Rev Infect Dis **13:**771-772.
3. **Augustin, D. K., Y. Song, M. S. Baek, Y. Sawa, G. Singh, B. Taylor, A. Rubio-Mills, J. L. Flanagan, J. P. Wiener-Kronish, and S. V. Lynch.** 2007. Presence or Absence of Lipopolysaccharide O Antigens Affects Type III Secretion by Pseudomonas aeruginosa. J. Bacteriol. **189:**2203-2209.
4. **Barnard, A., B. P. Mahon, J. Watkins, K. Redhead, and K. H. Mills.** 1996. Th1/Th2 cell dichotomy in acquired immunity to Bordetella pertussis: variables in the in vivo priming and in vitro cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0. Immunology **87:**372-380.
5. **Basler, M., J. Masin, R. Osicka, and P. Sebo.** 2006. Pore-forming and enzymatic activities of Bordetella pertussis adenylate cyclase toxin synergize in promoting lysis of monocytes. Infect Immun **74:**2207-2214.
6. **Baumler, A. J., R. M. Tsolis, T. A. Ficht, and L. G. Adams.** 1998. Evolution of host adaptation in Salmonella enterica. Infect Immun **66:**4579-4587.
7. **Belen, O., J. M. Campos, P. H. Cogen, and B. A. Jantausch.** 2003. Postsurgical meningitis caused by Bordetella bronchiseptica. Pediatr Infect Dis J **22:**380-381.
8. **Bengoechea, J. A., H. Najdenski, and M. Skurnik.** 2004. Lipopolysaccharide O antigen status of Yersinia enterocolitica O:8 is essential for virulence and absence of O antigen affects the expression of other Yersinia virulence factors. Molecular Microbiology **52:**451-469.
9. **Bergfors, E., B. Trollfors, J. Taranger, T. Lagergard, V. Sundh, and G. Zackrisson.** 1999. Parapertussis and pertussis: differences and similarities in incidence, clinical course, and antibody responses. Int J Infect Dis **3:**140-146.
10. **Binns, S. H., S. Dawson, A. J. Speakman, L. E. Cuevas, C. J. Gaskell, C. A. Hart, K. L. Morgan, and R. M. Gaskell.** 1999. Prevalence and risk factors for feline Bordetella bronchiseptica infection. Vet Rec **144:**575-580.
11. **Bisgard, K. M., F. B. Pascual, K. R. Ehresmann, C. A. Miller, C. Cianfrini, C. E. Jennings, C. A. Rebmann, J. Gabel, S. L. Schauer, and S. M. Lett.** 2004. Infant pertussis: who was the source? Pediatr Infect Dis J **23:**985-989.
12. **Bjornstad, O. N., and E. T. Harvill.** 2005. Evolution and emergence of Bordetella in humans. Trends Microbiol **13:**355-359.
13. **Burns, V. C., E. J. Pishko, A. Preston, D. J. Maskell, and E. T. Harvill.** 2003. Role of Bordetella O antigen in respiratory tract infection. Infect Immun **71:**86-94.
14. **Burns, V. C., E. J. Pishko, A. Preston, D. J. Maskell, and E. T. Harvill.** 2003. Role of Bordetella O Antigen in Respiratory Tract Infection. Infect. Immun. **71:**86-94.
15. **Cambier, J. C., S. B. Gauld, K. T. Merrell, and B. J. Vilen.** 2007. B-cell anergy: from transgenic models to naturally occurring anergic B cells? Nat Rev Immunol **7:**633-643.

16. **Caroff, M., and D. Karibian.** 2003. Structure of bacterial lipopolysaccharides. Carbohydrate Research **338:**2431.
17. **Casadevall, A., and L. A. Pirofski.** 2000. Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. Infect Immun **68:**6511-6518.
18. **CDC.** 2002. Pertussis--United States, 1997-2000. JAMA **287:**977-979.
19. **Cerutti, A.** 2008. The regulation of IgA class switching. Nat Rev Immunol **8:**421-434.
20. **Cherry, J. D.** 2005. The epidemiology of pertussis: a comparison of the epidemiology of the disease pertussis with the epidemiology of Bordetella pertussis infection. Pediatrics **115:**1422-1427.
21. **Cotter, P. A., M. H. Yuk, S. Mattoo, B. J. Akerley, J. Boschwitz, D. A. Relman, and J. F. Miller.** 1998. Filamentous hemagglutinin of Bordetella bronchiseptica is required for efficient establishment of tracheal colonization. Infect Immun **66:**5921-5929.
22. **Cummings, C. A., M. M. Brinig, P. W. Lepp, S. van de Pas, and D. A. Relman.** 2004. Bordetella species are distinguished by patterns of substantial gene loss and host adaptation. J Bacteriol **186:**1484-1492.
23. **David, S., R. van Furth, and F. R. Mooi.** 2004. Efficacies of whole cell and acellular pertussis vaccines against Bordetella parapertussis in a mouse model. Vaccine **22:**1892.
24. **David, S., R. van Furth, and F. R. Mooi.** 2004. Efficacies of whole cell and acellular pertussis vaccines against Bordetella parapertussis in a mouse model. Vaccine **22:**1892-1898.
25. **de Melker, H. E., J. F. Schellekens, S. E. Neppelenbroek, F. R. Mooi, H. C. Rumke, and M. A. Conyn-van Spaendonck.** 2000. Reemergence of pertussis in the highly vaccinated population of the Netherlands: observations on surveillance data. Emerg Infect Dis **6:**348-357.
26. **Decot, V., G. Woerly, M. Loyens, S. Loiseau, B. Quatannens, M. Capron, and D. Dombrowicz.** 2005. Heterogeneity of expression of IgA receptors by human, mouse, and rat eosinophils. J Immunol **174:**628-635.
27. **Deeb, B. J., R. F. DiGiacomo, B. L. Bernard, and S. M. Silbernagel.** 1990. Pasteurella multocida and Bordetella bronchiseptica infections in rabbits. J. Clin. Microbiol. **28:**70-75.
28. **Diavatopoulos, D. A., C. A. Cummings, L. M. Schouls, M. M. Brinig, D. A. Relman, and F. R. Mooi.** 2005. Bordetella pertussis, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of B. bronchiseptica. PLoS Pathog **1:**e45.
29. **Edwards, J. A., N. A. Groathouse, and S. Boitano.** 2005. Bordetella bronchiseptica adherence to cilia is mediated by multiple adhesin factors and blocked by surfactant protein A. Infect Immun **73:**3618-3626.
30. **Fujita, T.** 2002. Evolution of the lectin-complement pathway and its role in innate immunity. Nat Rev Immunol **2:**346-353.
31. **Gerlach, G., F. von Wintzingerode, B. Middendorf, and R. Gross.** 2001. Evolutionary trends in the genus Bordetella. Microbes Infect **3:**61-72.

32. **Goebel, E. M., D. N. Wolfe, K. Elder, S. Stibitz, and E. T. Harvill.** 2008. O-antigen Protects Bordetella parapertussis from Complement. Infect Immun.
33. **Gros, P., F. J. Milder, and B. J. C. Janssen.** 2008. Complement driven by conformational changes. Nat Rev Immunol **8:**48-58.
34. **Gueirard, P., C. Weber, A. Le Coustumier, and N. Guiso.** 1995. Human Bordetella bronchiseptica infection related to contact with infected animals: persistence of bacteria in host. J Clin Microbiol **33:**2002-2006.
35. **Harnden, A., C. Grant, T. Harrison, R. Perera, A. B. Brueggemann, R. Mayon-White, and D. Mant.** 2006. Whooping cough in school age children with persistent cough: prospective cohort study in primary care. Bmj **333:**174-177.
36. **Harvill, E. T., P. A. Cotter, M. H. Yuk, and J. F. Miller.** 1999. Probing the function of Bordetella bronchiseptica adenylate cyclase toxin by manipulating host immunity. Infect Immun **67:**1493-1500.
37. **Harvill, E. T., A. Preston, P. A. Cotter, A. G. Allen, D. J. Maskell, and J. F. Miller.** 2000. Multiple roles for Bordetella lipopolysaccharide molecules during respiratory tract infection. Infect Immun **68:**6720-6728.
38. **Harvill, E. T., A. Preston, P. A. Cotter, A. G. Allen, D. J. Maskell, and J. F. Miller.** 2000. Multiple Roles for Bordetella Lipopolysaccharide Molecules during Respiratory Tract Infection. Infect. Immun. **68:**6720-6728.
39. **Hellwig, S. M., A. B. van Spriel, J. F. Schellekens, F. R. Mooi, and J. G. van de Winkel.** 2001. Immunoglobulin A-mediated protection against Bordetella pertussis infection. Infect Immun **69:**4846-4850.
40. **Kirimanjeswara, G. S., L. M. Agosto, M. J. Kennett, O. N. Bjornstad, and E. T. Harvill.** 2005. Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of Bordetella pertussis. J Clin Invest **115:**3594-3601.
41. **Kirimanjeswara, G. S., P. B. Mann, and E. T. Harvill.** 2003. Role of antibodies in immunity to Bordetella infections. Infect Immun **71:**1719-1724.
42. **Kort, W. J., J. M. Hekking-Weijma, M. T. TenKate, V. Sorm, and R. VanStrik.** 1998. A microchip implant system as a method to determine body temperature of terminally ill rats and mice. Lab Anim **32:**260-269.
43. **Kuiken, T., E. C. Holmes, J. McCauley, G. F. Rimmelzwaan, C. S. Williams, and B. T. Grenfell.** 2006. Host species barriers to influenza virus infections. Science **312:**394-397.
44. **Leef, M., K. L. Elkins, J. Barbic, and R. D. Shahin.** 2000. Protective immunity to Bordetella pertussis requires both B cells and CD4(+) T cells for key functions other than specific antibody production. J Exp Med **191:**1841-1852.
45. **Liese, J. G., C. Renner, S. Stojanov, B. H. Belohradsky, and G. The Munich Vaccine Study.** 2003. Clinical and epidemiological picture of B pertussis and B parapertussis infections after introduction of acellular pertussis vaccines. Arch Dis Child **88:**684-687.
46. **Lohoff, M., and T. W. Mak.** 2005. Roles of interferon-regulatory factors in T-helper-cell differentiation. Nat Rev Immunol **5:**125-135.
47. **Lorenzo-Pajuelo, B., J. L. Villanueva, J. Rodriguez-Cuesta, N. Vergara-Irigaray, M. Bernabeu-Wittel, A. Garcia-Curiel, and G. Martinez de Tejada.** 2002. Cavitary pneumonia in an AIDS patient caused by an unusual Bordetella

bronchiseptica variant producing reduced amounts of pertactin and other major antigens. J Clin Microbiol **40:**3146-3154.

48. **Lugo, J. Z., S. Price, J. E. Miller, I. Ben-David, V. J. Merrill, P. Mancuso, J. B. Weinberg, and J. G. Younger.** 2007. Lipopolysaccharide O-antigen promotes persistent murine bacteremia. Shock **27:**186-191.
49. **Lysenko, E. S., A. J. Ratner, A. L. Nelson, and J. N. Weiser.** 2005. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. PLoS Pathog **1:**e1.
50. **Maixnerova, M.** 2003. The 2001 serological survey in the Czech Republic--parapertussis. Cent Eur J Public Health **11 Suppl:**S23-24.
51. **Maixnerova, M.** 2003. The 2001 serological survey in the Czech Republic--pertussis. Cent Eur J Public Health **11 Suppl:**S17-22.
52. **Mann, P. B., K. D. Elder, M. J. Kennett, and E. T. Harvill.** 2004. Toll-like receptor 4-dependent early elicited tumor necrosis factor alpha expression is critical for innate host defense against Bordetella bronchiseptica. Infect Immun **72:**6650-6658.
53. **Mann, P. B., M. J. Kennett, and E. T. Harvill.** 2004. Toll-like receptor 4 is critical to innate host defense in a murine model of bordetellosis. J Infect Dis **189:**833-836.
54. **Mann, P. B., D. Wolfe, E. Latz, D. Golenbock, A. Preston, and E. T. Harvill.** 2005. Comparative toll-like receptor 4-mediated innate host defense to Bordetella infection. Infect Immun **73:**8144-8152.
55. **Mattoo, S., and J. D. Cherry.** 2005. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin Microbiol Rev **18:**326-382.
56. **Mills, K. H., A. Barnard, J. Watkins, and K. Redhead.** 1993. Cell-mediated immunity to Bordetella pertussis: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect Immun **61:**399-410.
57. **Mira, A., R. Pushker, and F. Rodriguez-Valera.** 2006. The Neolithic revolution of bacterial genomes. Trends Microbiol **14:**200-206.
58. **Munford, R. S., and A. W. Varley.** 2006. Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria. PLoS Pathog **2:**e67.
59. **Nicosia, A., A. Bartoloni, M. Perugini, and R. Rappuoli.** 1987. Expression and immunological properties of the five subunits of pertussis toxin. Infect Immun **55:**963-967.
60. **Nicosia, A., and R. Rappuoli.** 1987. Promoter of the pertussis toxin operon and production of pertussis toxin. J Bacteriol **169:**2843-2846.
61. **Parkhill, J., M. Sebaihia, A. Preston, L. D. Murphy, N. Thomson, D. E. Harris, M. T. Holden, C. M. Churcher, S. D. Bentley, K. L. Mungall, A. M. Cerdeno-Tarraga, L. Temple, K. James, B. Harris, M. A. Quail, M. Achtman, R. Atkin, S. Baker, D. Basham, N. Bason, I. Cherevach, T. Chillingworth, M. Collins, A. Cronin, P. Davis, J. Doggett, T. Feltwell, A. Goble, N. Hamlin, H. Hauser, S. Holroyd, K. Jagels, S. Leather, S. Moule, H. Norberczak, S. O'Neil, D. Ormond, C. Price, E. Rabbinowitsch, S. Rutter, M. Sanders, D. Saunders, K. Seeger, S. Sharp, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, L. Unwin, S. Whitehead, B. G. Barrell, and D. J. Maskell.** 2003.

Comparative analysis of the genome sequences of Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Nat Genet **35:**32-40.
62. **Pier, G. B.** 2007. Pseudomonas aeruginosa lipopolysaccharide: A major virulence factor, initiator of inflammation and target for effective immunity. International Journal of Medical Microbiology **297:**277.
63. **Pilione, M. R., L. M. Agosto, M. J. Kennett, and E. T. Harvill.** 2006. CD11b is required for the resolution of inflammation induced by Bordetella bronchiseptica respiratory infection. Cell Microbiol **8:**758-768.
64. **Pilione, M. R., and E. T. Harvill.** 2006. The Bordetella bronchiseptica type III secretion system inhibits gamma interferon production that is required for efficient antibody-mediated bacterial clearance. Infect Immun **74:**1043-1049.
65. **Pishko, E. J., G. S. Kirimanjeswara, M. R. Pilione, L. Gopinathan, M. J. Kennett, and E. T. Harvill.** 2004. Antibody-mediated bacterial clearance from the lower respiratory tract of mice requires complement component C3. Eur J Immunol **34:**184-193.
66. **Preston, A., A. G. Allen, J. Cadisch, R. Thomas, K. Stevens, C. M. Churcher, K. L. Badcock, J. Parkhill, B. Barrell, and D. J. Maskell.** 1999. Genetic Basis for Lipopolysaccharide O-Antigen Biosynthesis in Bordetellae. Infect. Immun. **67:**3763-3767.
67. **Preston, A., A. G. Allen, J. Cadisch, R. Thomas, K. Stevens, C. M. Churcher, K. L. Badcock, J. Parkhill, B. Barrell, and D. J. Maskell.** 1999. Genetic basis for lipopolysaccharide O-antigen biosynthesis in bordetellae. Infect Immun **67:**3763-3767.
68. **Preston, A., E. Maxim, E. Toland, E. J. Pishko, E. T. Harvill, M. Caroff, and D. J. Maskell.** 2003. Bordetella bronchiseptica PagP is a Bvg-regulated lipid A palmitoyl transferase that is required for persistent colonization of the mouse respiratory tract. Mol Microbiol **48:**725-736.
69. **Preston, A., B. O. Petersen, J. O. Duus, J. Kubler-Kielb, G. Ben-Menachem, J. Li, and E. Vinogradov.** 2006. Complete Structures of Bordetella bronchiseptica and Bordetella parapertussis Lipopolysaccharides. J. Biol. Chem. **281:**18135-18144.
70. **Raynaud, C., K. L. Meibom, M.-A. Lety, I. Dubail, T. Candela, E. Frapy, and A. Charbit.** 2007. Role of the wbt Locus of Francisella tularensis in Lipopolysaccharide O-Antigen Biogenesis and Pathogenicity. Infect. Immun. **75:**536-541.
71. **Restif, O., and B. T. Grenfell.** 2006. Integrating life history and cross-immunity into the evolutionary dynamics of pathogens. Proc Biol Sci **273:**409-416.
72. **Sebastian, S., S. T. Dillon, J. G. Lynch, L. T. Blalock, E. Balon, K. T. Lee, L. E. Comstock, J. W. Conlan, E. J. Rubin, A. O. Tzianabos, and D. L. Kasper.** 2007. A defined O-antigen polysaccharide mutant of Francisella tularensis live vaccine strain has attenuated virulence while retaining its protective capacity. Infect Immun **75:**2591-2602.
73. **Shankar-Sinha, S., G. A. Valencia, B. K. Janes, J. K. Rosenberg, C. Whitfield, R. A. Bender, T. J. Standiford, and J. G. Younger.** 2004. The Klebsiella pneumoniae O Antigen Contributes to Bacteremia and Lethality during Murine Pneumonia. Infect. Immun. **72:**1423-1430.

74. **Skurnik, M., M. Biedzka-Sarek, P. S. Lubeck, T. Blom, J. A. Bengoechea, C. Perez-Gutierrez, P. Ahrens, and J. Hoorfar.** 2007. Characterization and Biological Role of the O-Polysaccharide Gene Cluster of Yersinia enterocolitica Serotype O:9. J. Bacteriol. **189:**7244-7253.
75. **Spencer, G.** September 7, 2007 2007, posting date. Background on Mouse as a Model Organism. [Online.]
76. **Stuart, L. M., and R. A. Ezekowitz.** 2008. Phagocytosis and comparative innate immunity: learning on the fly. Nat Rev Immunol **8:**131-141.
77. **Tan, M. W.** 2002. Cross-species infections and their analysis. Annu Rev Microbiol **56:**539-565.
78. **Trent, M. S., C. M. Stead, A. X. Tran, and J. V. Hankins.** 2006. Diversity of endotoxin and its impact on pathogenesis. J Endotoxin Res **12:**205-223.
79. **Trinchieri, G., and A. Sher.** 2007. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol **7:**179-190.
80. **van der Zee, A., F. Mooi, J. Van Embden, and J. Musser.** 1997. Molecular evolution and host adaptation of Bordetella spp.: phylogenetic analysis using multilocus enzyme electrophoresis and typing with three insertion sequences. J Bacteriol **179:**6609-6617.
81. **Vignali, D. A. A., L. W. Collison, and C. J. Workman.** 2008. How regulatory T cells work. Nat Rev Immunol **8:**523-532.
82. **Ward, J. I., J. D. Cherry, S. J. Chang, S. Partridge, H. Lee, J. Treanor, D. P. Greenberg, W. Keitel, S. Barenkamp, D. I. Bernstein, R. Edelman, and K. Edwards.** 2005. Efficacy of an acellular pertussis vaccine among adolescents and adults. N Engl J Med **353:**1555-1563.
83. **Watanabe, M., and M. Nagai.** 2004. Whooping cough due to Bordetella parapertussis: an unresolved problem. Expert Rev Anti Infect Ther **2:**447-454.
84. **Wolfe, D. N., E. M. Goebel, O. N. Bjornstad, O. Restif, and E. T. Harvill.** 2007. The O Antigen Enables Bordetella parapertussis To Avoid Bordetella pertussis-Induced Immunity. Infect. Immun. **75:**4972-4979.
85. **Wolfe, D. N., G. S. Kirimanjeswara, E. M. Goebel, and E. T. Harvill.** 2007. Comparative Role of Immunoglobulin A in Protective Immunity against the Bordetellae. Infect. Immun. **75:**4416-4422.
86. **Wolfe, D. N., G. S. Kirimanjeswara, and E. T. Harvill.** 2005. Clearance of Bordetella parapertussis from the lower respiratory tract requires humoral and cellular immunity. Infect Immun **73:**6508-6513.
87. **Wolfe, D. N., P. B. Mann, A. M. Buboltz, and E. T. Harvill.** 2007. Delayed role of tumor necrosis factor- alpha in overcoming the effects of pertussis toxin. J Infect Dis **196:**1228-1236.
88. **Woodard, D. R., L. A. Cone, and K. Fostvedt.** 1995. Bordetella bronchiseptica infection in patients with AIDS. Clin Infect Dis **20:**193-194.
89. **Woolfrey, B. F., and J. A. Moody.** 1991. Human infections associated with Bordetella bronchiseptica. Clin Microbiol Rev **4:**243-255.

Chapter 2: Immune Mediated Exclusion of *Bordetella bronchiseptica* from *B. pertussis* Immune Hosts

Abstract:

Bordetella bronchiseptica is a respiratory pathogen of a wide range of mammals but is only sporadically isolated from humans, a phenomenon often attributed to a receptor/ligand mediated host specificity. However, groups of very closely related strains from a single lineage of *B. bronchiseptica* have been isolated from a wide range of hosts. Furthermore, strains from all across the phylogenetic tree have been recovered from humans, indicating that many, if not all, strains of *B. bronchiseptica* are capable of infecting humans. Based on these observations and well-established theories of competition between pathogens that infect the same host population, we hypothesized that immunity to the endemic human pathogen *B. pertussis* protects against *B. bronchiseptica* challenge, potentially explaining the lack of efficient transmission of *B. bronchiseptica* in human populations. Using a murine model of infection, we showed that either infection or vaccination by *B. pertussis* induced protective immunity to three phylogenetically disparate strains of *B. bronchiseptica*. *B. pertussis*-induced antibodies recognized the prominent, shared surface antigen, pertactin, on *B. bronchiseptica*. Furthermore, vaccination with purified *B. pertussis*-derived pertactin, filamentous hemagglutinin or human acellular vaccine, Adacel, conferred protection against *B. bronchiseptica* challenge. These data support our model of immune-mediated exclusion of *B. bronchiseptica* from human populations, which also may explain why many of the sporadically observed *B. bronchiseptica* infections of humans have been associated with an immunocompromised state.

Introduction:

The classical bordetellae are closely related gram-negative bacterial species that cause respiratory tract infections in a variety of mammalian hosts (33). *Bordetella bronchiseptica* causes a chronic respiratory infection that can persist for the life of the animal (20, 33). This pathogen has been isolated from a diverse range of mammalian hosts including rodents, felines, canines, equines, bovines and primates. Although infections are often asymptomatic, this bacterium causes kennel cough in dogs, snuffles in rabbits and atrophic rhinitis in pigs (17, 20, 33). *B. pertussis* is a highly infectious human pathogen that induces protective immunity; however, this species can reinfect previously immunized individuals due to waning immunity (33).

Several phylogenetic analyses suggest that a *B. bronchiseptica*-like progenitor gave rise to the human-adapted *B. pertussis* (13, 17, 19, 35, 46). Interestingly, the adaptation of *B. pertussis* to humans was accompanied by a large-scale loss of genes, with little to no acquisition of new genes (7, 13, 19, 34, 35). Therefore, it is likely that *B. bronchiseptica* possesses all the genes necessary to infect humans. Although *B. bronchiseptica* does not express pertussis toxin, this toxin does not appear to be crucial for successful transmission within human populations, as *B. parapertussis* circulates despite the lack of this factor (2). However, unlike *B. parapertussis*, *B. bronchiseptica* is rarely found in humans and most previously described cases were associated with immunocompromised individuals (4, 14, 21, 29, 30, 52).

It is thought that host limitations of many pathogens may be due to receptor/ligand specificity between the pathogen and host (3, 8, 27, 31, 42). However, individual *B. bronchiseptica* strains have been shown to efficiently infect a wide range of mammals. Furthermore, *B. bronchiseptica* strains from across the phylogenetic tree have been isolated from humans (1, 4, 17, 21, 30, 51, 52), suggesting that *B. bronchiseptica* strains are not limited to non-human animals. However, *B. bronchiseptica* has not been associated with a whooping cough outbreak in humans. The rarity of *B. bronchiseptica* isolation from humans could be explained by basic ecological theories indicating that two closely related immunizing pathogens should not co-exist in the same host populations, as cross immunity will lead to immune-mediated competition (8, 19, 31, 38). Indeed, over 90% of humans in a population are seropositive for *B. pertussis*, indicating some level of immunity to this pathogen (28). Therefore, immune mediated competition conferred by *B. pertussis* may limit the circulation of *B. bronchiseptica* in human populations.

To examine immune mediated competition between *B. pertussis* and *B. bronchiseptica*, we quantitated the effects of *B. pertussis*-induced immunity on the ability of *B. bronchiseptica* to successfully infect mice (22, 25, 45). We show here that *B. pertussis* infection- and vaccine-induced immunity protects against *B. bronchiseptica* colonization. This protection is dependent on cross reacting antibodies that recognize shared antigens and clear *B. bronchiseptica* via Fcγ receptors and complement. In

addition, immunization with *B. pertussis*-derived antigens, specifically pertactin and filamentous hemagglutinin, was sufficient to induce protective immunity to *B. bronchiseptica*. Together, these data may explain, in part, why *B. bronchiseptica* is found in such a wide host range but is seldom observed in humans, the lone host species of *B. pertussis.*

Materials and Methods:

Bacterial Growth: *Bordetella pertussis* strain 536, a streptomycin resistant derivative of Tohoma I (41), *B. bronchiseptica* strain RB50 (11), and *B. bronchiseptica* strain RB50G, a gentamicin-resistant derivative of RB50 (12), have been previously described. *B. bronchiseptica* strain SP5, an isogenic mutant of RB50 with a partial deletion of pertactin (Δ*prn*) (18), and *B. bronchiseptica* strain RBX9, an isogenic mutant of RB50 lacking filamentous hemagglutinin (Δ*fha*) (12) have been previously described. Human isolates of *B. bronchiseptica,* strains A345 (a.k.a. B2493 and GA96-01) and M0149 (a.k.a. D444 and B2494), were received from the CDC and multi-locus sequence typed as previously described (17). All strains were maintained on Bordet-Gengou (BG) Agar (Difco, Sparks, MD) with 10% sheep's blood (Hema Resources, Aurora, OR) with 20 µg/ml of streptomycin or gentamicin. Bacteria were grown overnight at 37 ^{o}C in Stainer-Scholte broth (24, 40, 47) to mid-exponential phase and diluted in phosphate buffered saline (PBS, Omnipur, Gibbstown, NJ) to the indicated concentration.

Animal Care and Housing: 4 to 6 week old C57BL/6, µMT and RAG2 $^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). IgA $^{-/-}$ mice were obtained from Dr. Innocent Mbawuike (53). C3 $^{-/-}$ mice were obtained from Dr. Rick Wetsel (10). Mice lacking Fcγ Receptor I, II, and III (FcγR$^{-/-}$) were obtained from Taconic (Hudson, NY). All mice were maintained and bred in Pennsylvania State University approved housing facilities and were closely monitored in accordance with institutional policies and IACUC regulations.

Inoculation and Vaccination of Mice: For inoculation, mice were lightly sedated by a flow of 5% isoflorane in oxygen and a 50 µl inoculum containing 5 x 10^5 CFU was pipetted gently onto the external nares. This method of inhalation inoculation reliably distributes the bacteria throughout the respiratory tract (22). For vaccination, the mice were intraperitoneally (i.p.) injected with 1 x 10^8 heat killed *B. pertussis* in 1 ml of PBS (26), 40 µg of purified pertactin 1 (23) or 5 µg of filamentous hemagglutinin (Sigma, St. Louis, MO) in 200 µL of PBS with Imject Alum (Pierce, Rockford, IL), or subcutaneously injected with 0.5 ml of 1:5 dilutent of the 5-component human vaccine, Adacel (aP) (Sanofi-Pasteur, Swiftwater, PA) in PBS with Imject Alum on Day 0 and Day 14. Mice were euthanized by CO_2 inhalation and lungs and trachea were excised, homogenized and serially diluted in PBS. Aliquots were then plated on BG Agar with appropriate antibiotics and the resultant colonies were counted two days later.

Antibiotic treatment and depletion of Immune Factors: For reinfection experiments, 1% gentamicin (Sigma, St. Louis, MO) was administrated in the drinking water for 3 days beginning on day 23 post-*B. pertussis* inoculation (48). Mice were then given untreated water for 2 days prior to challenge with RB50G. Previous studies have

shown that gentimicin treatment does not hinder *B. bronchiseptica* strain RB50G colonization of the murine respiratory tract (48). Neutrophils were depleted by i.p. injection of 1 mg of mAb from the hybridoma RB6-8C5 (αLy-6G) 48 hours prior to bacterial inoculation (26). Complement was depleted by two i.p. injections of 5 units of Cobra Venom Factor (CVF, Sigma, St. Louis, MO) 24 and 22 hours prior to inoculation (39).

Generation and Passive Transfer of Immune Serum: Convalescent mice were generated by inoculating mice with the indicated bacteria and allowing the mice to recover for 28 days (26). Pooled serum was collected via post-mortem cardiac puncture from wild type convalescent or naive mice. To induce a higher titer of *B. pertussis*-specific antibodies, *B. pertussis*-inoculated mice were allowed to convalesce for 28 days followed by a second challenge with *B. pertussis* and subsequent cardiac puncture 3 days post secondary inoculation. To generate complement deficient serum, sera were heat-inactivated at 65°C for 30 minutes prior to passive transfer. Passive transfer experiments used 200μL of serum i.p. injected at the time of inoculation (25, 26).

Western Blot Analysis: Western Blots were performed on lysates of 1×10^7 CFU of *B. pertussis,* or *B. bronchiseptica* in 10μL or 1:2 dilution of Adacel (aP) (Sanofi-Pasteur, Swiftwater, PA) in 20 μL, as indicated. Lysates were run on 7% SDS-PAGE gels under denaturing conditions. PVDF membranes were probed with a 1:50 dilution of primary antibodies from aP-vaccinated serum and a 1:10,000 dilution of goat anti-mouse Ig (H + L) HRP-conjugated (Southern Biotech, Birmingham, AL) was used as the detector antibody. The membrane was visualized with ECL Western Blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis: Student's two-tailed t-test was used to determine statistical significance between experimental groups. *P*-values of ≤0.05 were considered significant. Error bars represent SEM.

Results:

Immunity to *B. pertussis* protects against *B. bronchiseptica* challenge. To test the hypothesis that *B. pertussis*-induced immunity protects against *B. bronchiseptica* colonization of the lower respiratory tract, wild type mice were infected with live *B. pertussis* or vaccinated with heat killed *B. pertussis* and allowed to recover for at least 28 days. By this time point, bacterial numbers in the respiratory tract were reduced to approximately 10^2 or fewer CFU and a strong immune response had been induced (25). *B. pertussis*-vaccinated, -previously infected, or naïve mice were left untreated or were gentimicin treated to clear any remnant bacteria and then challenged with a gentimicin resistant strain of *B. bronchiseptica* and the bacterial load was enumerated on Days 3, 7, 10 and 14 post-challenge. Approximately 10^4 CFU in the trachea and 10^6 CFU in the lungs were recovered on both Days 3 and 7 post-challenge in naïve mice (Figure 2.1).

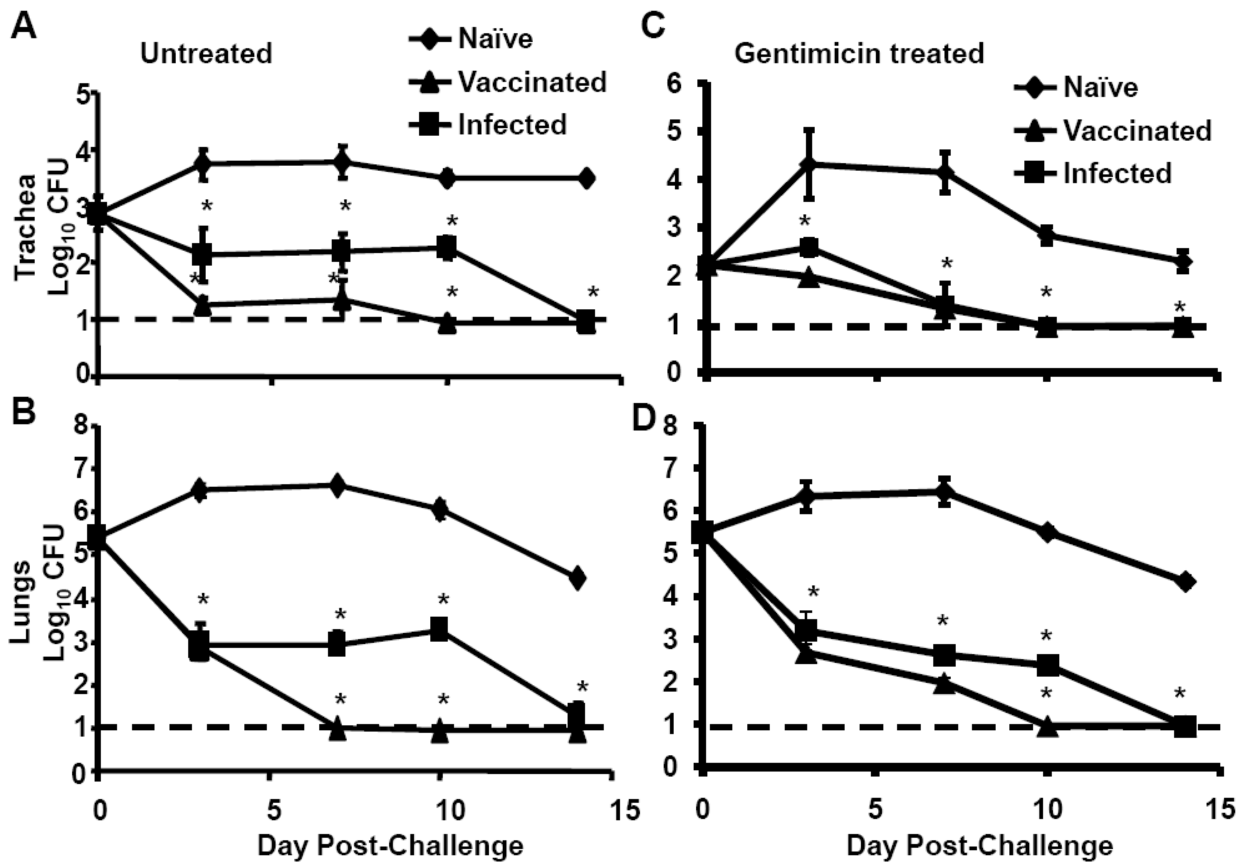

Figure 2.1: *B. bronchiseptica* numbers in the lower respiratory tract of naïve and *B. pertussis* immune mice over time. Groups of 3 to 4 C57BL/6 mice were left uninoculated (♦), inoculated with 5×10^5 CFU of *B. pertussis* (■), or vaccinated with 2 i.p. injections of 10^8 heat killed *B. pertussis* on Days 0 and 14 (▲). Mice were then left untreated (A and B) or were gentimicin-treated (C and D). Mice were challenged with a gentimicin^R strain of *B. bronchiseptica* on Day 28 and sacrificed at the indicated day post-challenge for quantification of bacterial load in (A and C) the trachea and (B and D) the lungs. Bacterial numbers are represented as the mean Log_{10} CFU +/- SEM. Dashed line represents the lower limit of detection. * indicates statistical difference (P value < 0.05) between naïve and treated groups.

However, *B. bronchiseptica* numbers were reduced approximately 100-fold to 10^2 CFU in the trachea and 3,000-fold to 10^3 CFU in the lungs by Day 3 post-challenge in immunized mice as compared to naïve animals (Figure 2.1). By Day 7 post-challenge, previously infected mice retained approximately 10^2 CFU of *B. bronchiseptica* in the trachea, and 10^3 CFU of *B. bronchiseptica* in the lungs, and carried these low loads of *B. bronchiseptica* for approximately 2 weeks, until bacteria were undetectable on Day 14 post-challenge. Vaccinated mice cleared *B. bronchiseptica* from the trachea and lungs by Day 7 post-inoculation (Figure 2.1). These data support the hypothesis that immunity to *B. pertussis* protects against a *B. bronchiseptica* challenge. Because a very large and significant effect of prior exposure to *B. pertussis* on colonization by *B. bronchiseptica* was observed on Day 3 post-challenge, subsequent experiments were carried out at this timepoint.

***B. pertussis*-induced immunity requires B cells, but not IgA, for protection against *B. bronchiseptica* challenge in the lungs.** Since immunization with *B. pertussis* led to protection against *B. bronchiseptica*, we sought to determine if this was mediated by the adaptive immune response. *B. pertussis*-immunized $RAG^{-/-}$ mice were unable to reduce *B. bronchiseptica* numbers, indicating a role for adaptive immunity in protection (data not shown). We hypothesized that *B. pertussis*-induced antibodies could recognize *B. bronchiseptica* antigens and mediate the clearance of this pathogen. Supporting this hypothesis, there was no difference in bacterial load in the trachea or the lungs between immunized or naïve μMT mice on Day 3 post *B. bronchiseptica* challenge (Figure 2.2). These data indicate that B cells, and the antibodies they produce (25), are required for cross protection.

We further hypothesized that the major mucosal antibody, IgA, would be the primary protective antibody (48). $IgA^{-/-}$ mice were vaccinated or infected with *B. pertussis* and allowed to convalesce for 28 days. The treated or naïve mice were then challenged with *B. bronchiseptica* and dissected Day 3 post-inoculation. The bacterial load in the lungs of $IgA^{-/-}$ mice was not significantly different from similarly treated wild type

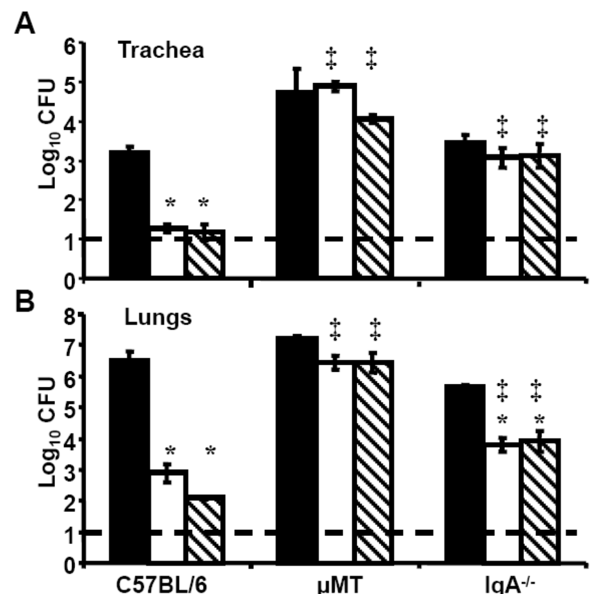

Figure 2.2: *B. bronchiseptica* numbers in the lower respiratory tract of immunized B-cell and IgA deficient mice. Naïve (black bars), *B. pertussis*-vaccinated (white bars), or *B. pertussis*-convalescent (hatched bars) C57BL/6, μMT and $IgA^{-/-}$ mice were dissected Day 3 post gentimicin^R *B. bronchiseptica* challenge. Bacterial numbers in the (A) trachea and (B) lungs are represented as the mean Log_{10} CFU +/- SEM. Dashed line indicates the lower limit of detection. * indicates statistical difference (*P* value < 0.05) between naïve and treated groups. ‡ indicates statistical difference (*P* value < 0.05) between mutant and similarly treated wild type groups.

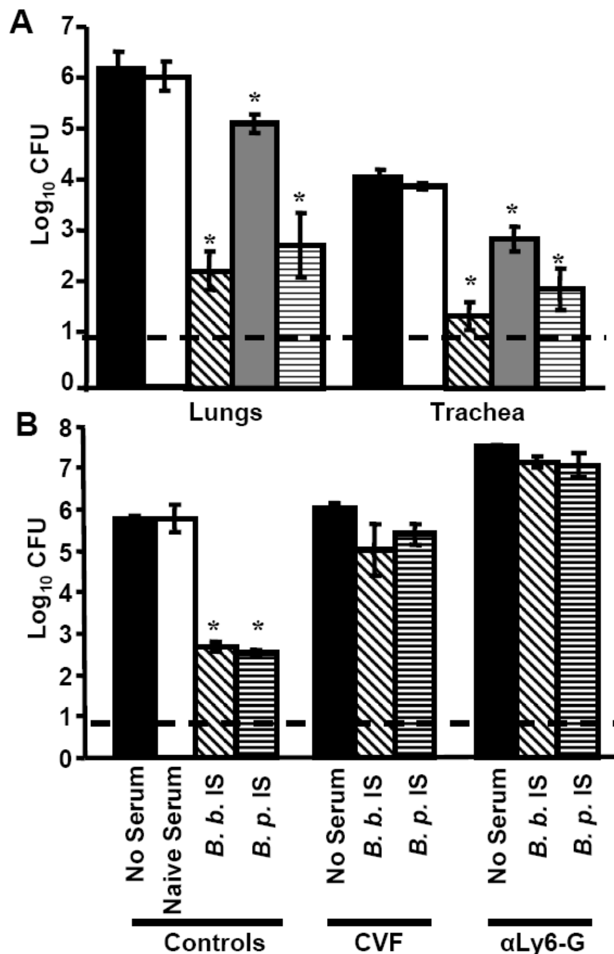

Figure 2.3: Effect of Complement and Neutrophils on *B. pertussis* serum antibody-mediated clearance of *B. bronchiseptica*. (A) Wild type mice infected with *B. bronchiseptica* were untreated (black bars) or i.p. injected with naïve serum (white bars), *B. bronchiseptica*-immune serum (hatched bars), *B. pertussis*-immune serum titer 400 (gray bars), or *B. pertussis*-immune serum titer 6400 (horizontal lined bars) and then dissected Day 3 post-inoculation. (B) Wild type mice were complement (CVF) or neutrophil (αLy6-G) depleted, then inoculated with *B. bronchiseptica*, i.p. injected with the indicated serum and then dissected on Day 2 post-inoculation. *B.b.* indicates *B. bronchiseptica*-immune serum; *B.p.* indicates *B. pertussis*-immune serum titer 6400. Bacterial numbers are represented as the mean Log_{10} CFU +/- SEM. Dashed line indicates the lower limit of detection. * indicates P value of < 0.05.

mice (Figure 2.2). Consistent with previous findings, immunized $IgA^{-/-}$ mice were not protected against *B. bronchiseptica* challenge in the trachea (48). Thus, IgA is required for *B. pertussis*-immune-mediated protection against *B. bronchiseptica* in the trachea, but not the lungs.

***B. pertussis*-induced antibodies require complement and neutrophils to reduce *B. bronchiseptica* colonization.** To determine if *B. pertussis*-induced antibodies can clear *B. bronchiseptica* upon passive transfer into naïve mice, wild type mice were inoculated with *B. bronchiseptica* and immediately i.p. injected with 200 µL of naïve, *B. bronchiseptica*- or *B. pertussis*-induced immune serum. The mice were then dissected on Day 3 post-inoculation for bacterial enumeration. Compared to untreated mice, naïve serum had no effect on bacterial numbers (Figure 2.3A). Transfer of *B. bronchiseptica*-immune serum resulted in 5,000 fold lower bacterial numbers by Day 3 post-inoculation (Figure 2.3). *B. pertussis*-immune serum (titer ~ 400) reduced bacterial numbers in the lungs by approximately 10-fold (Figure 2.3). *B. pertussis*-immune serum with a titer of 6400, a titer similar to that of *B. bronchiseptica*-induced immune serum, decreased *B. bronchiseptica* numbers by 5,000 fold, an effect similar to that of *B. bronchiseptica*-immune serum (Figure 2.3). Together, these data suggest that *B. pertussis*-induced antibodies are able to clear *B. bronchiseptica* from the lower respiratory tract in a dose-dependent manner.

Our previous work showed that antibodies induced by *B. bronchiseptica* infection clear this bacterium from the lungs of mice via complement cascade and neutrophils (36, 49). Thus, we hypothesized that *B. pertussis*-induced antibodies also clear *B. bronchiseptica* via the complement cascade and/or neutrophil mediated clearance. To test this hypothesis, mice were CVF treated to deplete complement or were treated with αLy-6G monoclonal antibody to deplete neutrophils prior to inoculation with *B. bronchiseptica*. While *B. pertussis*-immune serum reduced *B. bronchiseptica* numbers in untreated mice, it did not reduce bacterial numbers in the lungs of CVF or αLy-6G treated mice, indicating that both complement and neutrophils are required (Figure 2.3B). In addition, all αLy-6G treated mice were moribund by Day 2 post-inoculation, indicating that *B. pertussis*-specific antibodies did not protect against the rapid virulence of *B. bronchiseptica* in animals lacking neutrophils.

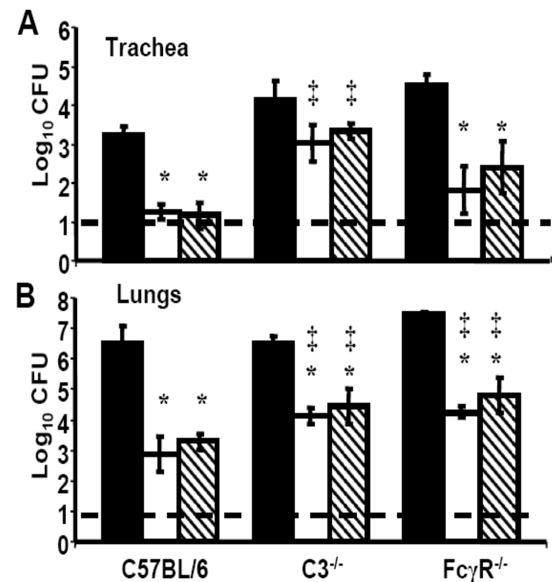

Figure 2.4: Clearance of *B. bronchiseptica* from the lower respiratory tract in *B. pertussis*-immunized $C3^{-/-}$ and $Fc\gamma R^{-/-}$ mice. Naïve (black bars), *B. pertussis*-vaccinated (white bars), or *B. pertussis*-convalescent (hatched bars) mice were dissected Day 3 post gentimicin^R *B. bronchiseptica* inoculation. Bacterial numbers in the (A) trachea and (B) lungs are represented as the mean Log_{10} CFU +/- SEM. Dashed line indicates the lower limit of detection. * indicates statistical difference (P value <0.05) between naïve and treated mouse. ‡ indicates statistical difference (P value < 0.05) between mutant and similarly treated wild type mouse.

To determine if complement and Fcγ Receptors are required for generation of an efficient adaptive immune response, $C3^{-/-}$ mice, which lack the enzyme required for both the classical and the alternative complement cascades, and $Fc\gamma R^{-/-}$ mice, which lack Fcγ Receptors (I, II, and III) specific for the Fc region of IgG antibodies, were immunized with *B. pertussis* and then challenged 28 days later with *B. bronchiseptica*. Both $C3^{-/-}$ and $Fc\gamma R^{-/-}$ mice that were previously vaccinated or infected with *B. pertussis* showed approximately a 300 fold decrease in *B. bronchiseptica* bacterial load in the lungs by Day 3 post-inoculation when compared to naïve mice (Figure 2.4). However, both $C3^{-/-}$ and $Fc\gamma R^{-/-}$ mice were defective in bacterial clearance in the lungs as compared to similarly treated wild type mice. $C3^{-/-}$ mice also showed this defect in clearance in the trachea, however, bacterial load in the trachea of $Fc\gamma R^{-/-}$ mice was similar to wild type mice (Figure 2.4). These data indicate that complement enhances efficient clearance throughout the respiratory tract, while Fcγ Receptor-bearing cells contribute to efficient clearance in the lungs but not the trachea. Therefore, both the complement cascade and Fcγ Receptor-bearing cells aid in *B. pertussis*-induced immune-mediated clearance of *B. bronchiseptica*.

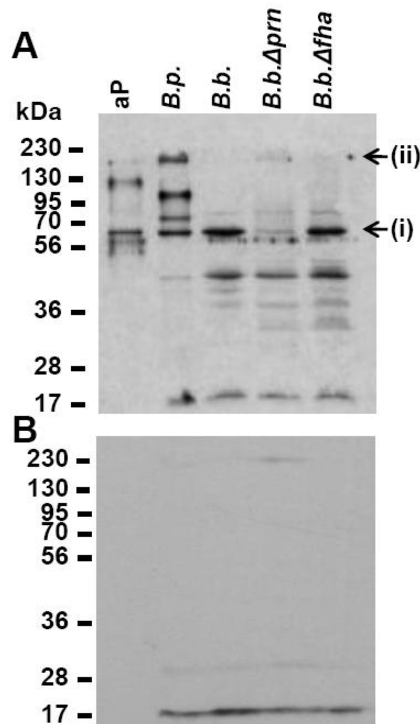

Figure 2.5: Analysis of cross-reacting antigens between *B. pertussis* and *B. bronchiseptica*. Western Blots were performed to determine *B. bronchiseptica* antigens recognized by (A) aP-induced or (B) naïve serum. Lysates of Adacel (aP), *B. pertussis* (*B.p.*), *B. bronchiseptica* strain RB50 (*B.b.*), *B. bronchiseptica* strain SP5 (*B.b.Δprn*), or *B. bronchiseptica* strain RBX9 (*B.b.Δfha*) were loaded in the indicated wells and separated by SDS-PAGE gel. Proteins were transferred to PVDF membrane, and then probed with *B. pertussis*-vaccine induced immune serum. (i) indicates the 65-70 KDa band. (ii) indicates the 220-230 KDa band.

B. pertussis*-induced antibodies recognize shared antigens on *B. pertussis* and *B. bronchiseptica. Since passive immunization with *B. pertussis*-induced antibodies can control *B. bronchiseptica* infection in the lower respiratory tract, we examined recognition of *B. bronchiseptica* antigens by *B. pertussis*-induced antibodies. Western Blot analysis of *B. pertussis, B. bronchiseptica* and aP lysates probed with aP-induced serum showed that *B. pertussis*-induced antibodies were cross reactive with *B. bronchiseptica* antigens (Figure 2.5A). The size of one of the cross-reactive bands at approximately 63-70 KDa suggested that the band was pertactin, an antigenic protein known to induce protective immunity against *B. pertussis* infections (23, 37, 45). To test this, an isogenic *B. bronchiseptica* mutant lacking pertactin (*Δprn*) and an isogenic *B. bronchiseptica* mutant lacking filamentous hemagglutinin (*Δfha*), were also probed with *B. pertussis*-induced antibodies. The 63-70 KDa (i) band was visible in *B. pertussis* and *B. bronchiseptica* lysates, it was not visible in *B. bronchisepticaΔprn*, suggesting that this band was pertactin (Figure 2.5A). The large 200 KDa (ii) band was not visible in the lysate of *B. bronchisepticaΔfha*, suggesting that this cross-reactive antigen is filamentous hemagglutinin (Figure 2.5A). Several other antigens were also cross-reactive, indicating that *B. pertussis* and *B. bronchiseptica* possess additional shared antigens. There was little to no recognition of these lysates when probed with naïve serum at the same dilution (Figure 2.5B)

Immunization with *B. pertussis*-derived antigens is protective against *B. bronchiseptica* challenge. Since we could identify two shared antigens based on size, we went on to determine if immunity to pertactin or filamentous hemagglutinin contributed to *B. pertussis*-induced immunity to *B. bronchiseptica*. Wild type mice were immunized with purified *B. pertussis*-derived pertactin, filamentous hemagglutinin, or aP vaccine, which contains these two shared antigens. These mice were then challenged with *B. pertussis* or *B. bronchiseptica* and dissected 3 days later to quantify bacterial numbers. Compared to adjuvant-treated control mice, all

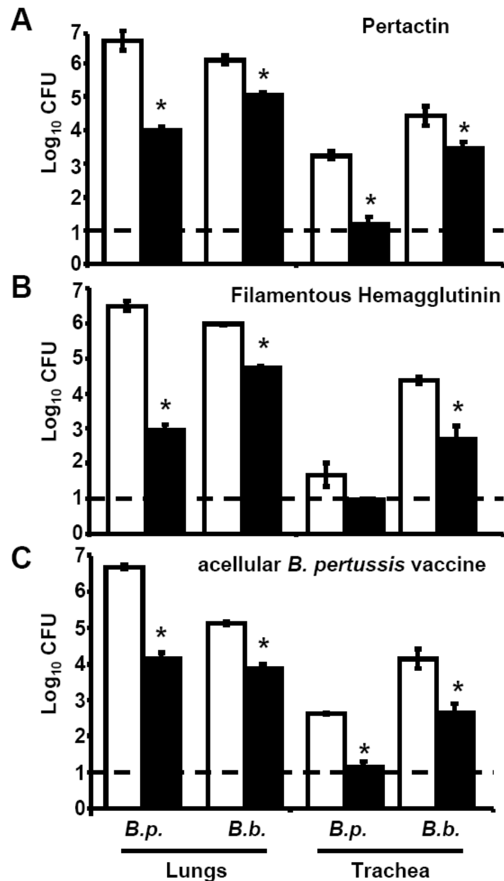

Figure 2.6: Effect of vaccination with *B. pertussis*-derived antigens on *B. bronchiseptica* colonization in the lower respiratory tract. Groups of 3-4 C57BL/6 mice were vaccinated with (A) 40 µg PRN, (B) 5 µg FHA or (C) 0.5 mL of 1:5 diluent of Adacel in PBS and Imject Alum on Days 0 and 14. Adjuvant-only control (white bars) and protein with adjuvant (black bars) vaccinated mice were challenged on Day 28 with *B. pertussis* (*B. p.*) or *B. bronchiseptica* (*B. b.*) and dissected Day 3 post-challenge. Bacterial numbers are represented as the mean Log_{10} CFU +/- SEM. Dashed line indicates the lower limit of detection. * indicates statistical difference (P value of < 0.05) between adjuvant only and protein with adjuvant treated groups.

immunized mice challenged with *B. pertussis* showed more than 99% reduction in bacterial load in the lungs (Figure 2.6A, B and C), consistent with previous findings (23). Immunized mice challenged with *B. bronchiseptica* showed a 10 to 30-fold decrease in the trachea and a 10-fold decrease in colonization in the lungs as compared to adjuvant-treated mice (Figure 2.6). These data indicate that immunization with *B. pertussis*-derived antigens induces an immune response which is effective against *B. bronchiseptica*.

***B. pertussis*-induced immunity protects against human isolates of *B. bronchiseptica*.** To determine if *B. pertussis*-induced immunity is sufficient to protect against recent human clinical isolates of *B. bronchiseptica*, wild type mice were immunized with *B. pertussis* and challenged with two *B. bronchiseptica* Complex IV isolates (17), strain M0149 and strain 345. These strains, which are divergent from the prototypical *B. bronchiseptica* Complex I strain, RB50, are more closely related to *B. pertussis* and are both from human-associated lineages (17). Since the normal 5 x 10^5 CFU dose was lethal to naïve mice, strain 345 was delivered in an inocula of 2 x 10^5 CFU. These bacteria

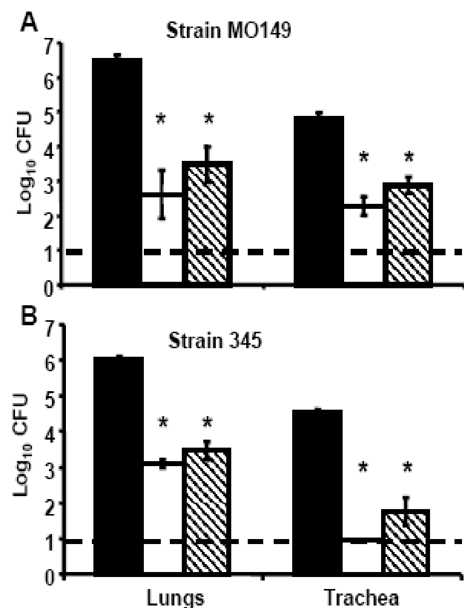

Figure 2.7: Human isolated, Complex IV *B. bronchiseptica* colonization of *B. pertussis*-immune mice. Groups of 4 naïve (black bars), *B. pertussis*-vaccinated (white bars) or *B. pertussis*-convalescent (hatched bars) C57BL/6 mice were challenged with *B. bronchiseptica* strain (A) M0149 or (B) 345 and dissected Day 3 post-challenge. Bacterial numbers are represented as the mean Log_{10} CFU +/- SEM. Dashed line indicates the lower limit of detection. * indicates statistical difference (P value of < 0.05) between naïve and treated groups

grew to 10^6 in the lungs and 10^5 in the trachea of naïve mice. In contrast, *B. pertussis*-immunized mice were able to reduce both strain M0149 and strain 345 to 10^3 in the lungs and 10^2 in the trachea (Figure 2.7A and B) by Day 3 post-challenge; indicating that immunity to *B. pertussis* is sufficient to control *B. bronchiseptica* strains recently isolated from human individuals.

Discussion:

We hypothesize that immunity to the human pathogen *B. pertussis* protects against *B. bronchiseptica* challenge, a pathogen that is endemic among a wide range of mammals (17, 33) but is rarely found in human populations. Immunity induced by *B. pertussis* infection or vaccination significantly reduced *B. bronchiseptica* numbers in the lower respiratory tract compared to naïve mice (Figure 2.1). Antibodies induced by *B. pertussis* immunization cross-reacted with *B. bronchiseptica* pertactin and filamentous hemagglutinin and mediated protection (Figures 2.2, 2.3, and 2.5). Immunization with purified pertactin or filamentous hemagglutinin derived from *B. pertussis* also conferred protection against *B. bronchiseptica* (Figure 2.6). Together, these data indicate that *B. pertussis*-induced immunity provides protection against *B. bronchiseptica* and suggests that immunity to *B. pertussis* may limit the ability of *B. bronchiseptica* to circulate in human populations.

Interestingly, the major mucosal antibody, IgA, was not required for *B. pertussis*-induced protection against *B. bronchiseptica* in the lungs, but was required in the trachea (Figure 2.2). This is consistent with the fact that IgA has been shown to be protective against *B. bronchiseptica* in the nasal cavity and trachea but not the lungs (48). Since phagocytic cells are found in greater numbers in the lungs, as opposed to the trachea, this could explain why other isotypes, which aid in phagocytosis, are more important in the lungs than IgA. Consistent with this model, *B. pertussis*-immune FcγR$^{-/-}$ mice show the opposite phenotype, compared to IgA$^{-/-}$ mice, with a defect in bacterial control in the lungs, but not the trachea (Figure 2.4). This suggests that opsonizing isotypes and subsequent phagocytosis are more important to protection in the lungs.

B. bronchiseptica infections are occasionally observed in humans. Many of the reported infections, however, have been associated with immunocompromised individuals (1, 4, 14, 21, 29, 30, 51, 52). Since clinical literature primarily reports infections associated with substantial disease, it is difficult to estimate from these data how frequently *B. bronchiseptica* may cross-over from companion animals to immunocompetent humans previously exposed to *B. pertussis,* particularly if these infections are asymptomatic. These would be expected to be minor, self-limiting infections that are unlikely to be diagnosed. Even if accurately diagnosed, the CDC specifically excludes *B. bronchiseptica* from the list of reportable agents (9, 21, 43). In fact, even *B. pertussis*, which is reportable to the CDC, is believed to be greatly under-reported (<1% reported) (15). Therefore it is difficult to estimate the prevalence of *B. bronchiseptica* infections in human (5, 16, 50).

Our data show that *B. bronchiseptica* colonization is inhibited by the immune response induced by *B. pertussis* infection, whole cell vaccination and acellular vaccination (Figure 2.1, 2.6 and 2.7). Most humans in developed countries are vaccinated against *B. pertussis* at a very early age. In unvaccinated populations, most individuals are infected by *B. pertussis* by the age of 5 (33). Thus, most humans have

developed some form of immunity to *B. pertussis* early in life (32). Indeed, recent estimates have suggested that more than 90% of a population may be seropositive for *B. pertussis*-specific antibodies (15). Once *B. pertussis* became highly endemic, the ability of *B. bronchiseptica* to circulate in human populations may have been limited to individuals with waning or non-functioning immunity, inhibiting the establishment of a successful chain of transmission among humans (5). Supporting this model, transmission of *B. bronchiseptica* between humans has not been reported.

An interesting disparity between humans and other mammalian hosts is the inability of the classical bordetellae to persist in the upper respiratory tract of humans (33). This absence may be due to a lack of receptor/ligand interactions with host cells in the upper respiratory tract of humans (44) or the resident microbial flora of the upper respiratory tract of humans may compete with the bordetellea. To exploit the human host, the bordetellae may have been limited to an available niche in the lower respiratory tract. This model could explain *B. pertussis*' apparent loss of ability to persist in the nasal cavity and also could explain why immunity to *B. pertussis* is not protective against *B. bronchiseptica* in the nasal cavity of mice (data not shown). This model could also explain the occurance of severe disease in, but not transmission from, immunocompromised patients, observations difficult to reconcile with receptor/ligand type specificity. While some level of host specificity for *B. pertussis* in humans exist, the data presented herein using a murine model provides suggestive evidence that immune mediated pressures can provide an alternative, though not mutually exclusive, explanation for the apparent limitations for successful colonization by *B. bronchiseptica* within human populations.

Rapid transmission allows *B. pertussis* to circulate efficiently, and large populations have a high prevalence of detectable immunity against *B. pertussis* (6, 34). The ability of *B. pertussis* to avoid rapid antibody-mediated clearance (25) likely enhances its ability to infect hosts with measurable immunity, and may allow this bacterium to circulate even in immunized populations. *B. bronchiseptica* is susceptible to both rapid antibody mediated clearance (25) and *B. pertussis*-induced immunity. Together these data suggest an alternative explanation for why *B. bronchiseptica* is common in many of the mammals around us, but is rarely described in humans. While some level of receptor/ligand specificity of *Bordetella* species or lineages may exist, these data suggest that strong immune mediated pressures may also contribute to the apparent exclusion of *B. bronchiseptica* from human populations. This alternative model is consistent with much of the existing experimental and clinical data (4, 14, 21, 29, 30, 52). From this model, we can predict that *B. bronchiseptica* infections occur in humans, but may be limited by pre-existing immunity to *B. pertussis* and, therefore, are unreported.

References:

1. **Amador, C., E. Chiner, J. L. Calpe, V. Ortiz de la Table, C. Martinez, and F. Pasquau.** 1991. Pneumonia due to Bordetella bronchiseptica in a patient with AIDS. Rev Infect Dis 13:771-772.
2. **Arico, B., and R. Rappuoli.** 1987. Bordetella parapertussis and Bordetella bronchiseptica contain transcriptionally silent pertussis toxin genes. J Bacteriol 169:2847-2853.
3. **Baumler, A. J., R. M. Tsolis, T. A. Ficht, and L. G. Adams.** 1998. Evolution of host adaptation in Salmonella enterica. Infect Immun 66:4579-4587.
4. **Belen, O., J. M. Campos, P. H. Cogen, and B. A. Jantausch.** 2003. Postsurgical meningitis caused by Bordetella bronchiseptica. Pediatr Infect Dis J 22:380-381.
5. **Bjornstad, O. N., and E. T. Harvill.** 2005. Evolution and emergence of Bordetella in humans. Trends Microbiol 13:355-359.
6. **Bocquet-appel, J. P.** 2002. Paleoanthropological Traces of a Neolithic Demographic Transition. Current Anthropology 43:637-650.
7. **Brinig, M. M., C. A. Cummings, G. N. Sanden, P. Stefanelli, A. Lawrence, and D. A. Relman.** 2006. Significant gene order and expression differences in Bordetella pertussis despite limited gene content variation. J Bacteriol 188:2375-2382.
8. **Casadevall, A., and L. A. Pirofski.** 2000. Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. Infect Immun 68:6511-6518.
9. **CDC.** 2002. Pertussis--United States, 1997-2000. JAMA 287:977-979.
10. **Circolo, A., G. Garnier, W. Fukuda, X. Wang, T. Hidvegi, A. J. Szalai, D. E. Briles, J. E. Volanakis, R. A. Wetsel, and H. R. Colten.** 1999. Genetic disruption of the murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA. Immunopharmacology 42:135-149.
11. **Cotter, P. A., and J. F. Miller.** 1994. BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of Bordetella bronchiseptica in a rabbit model. Infect Immun 62:3381-3390.
12. **Cotter, P. A., M. H. Yuk, S. Mattoo, B. J. Akerley, J. Boschwitz, D. A. Relman, and J. F. Miller.** 1998. Filamentous Hemagglutinin of Bordetella bronchiseptica Is Required for Efficient Establishment of Tracheal Colonization. Infect. Immun. 66:5921-5929.
13. **Cummings, C. A., M. M. Brinig, P. W. Lepp, S. van de Pas, and D. A. Relman.** 2004. Bordetella species are distinguished by patterns of substantial gene loss and host adaptation. J Bacteriol 186:1484-1492.

14. **De Jesus-Berrios, Y., S. Gonzalez, and M. Sante.** 2005. Respiratory pathogens in bronchoalveolar lavage in a Puerto Rican population infected with the human immunodeficiency virus. P R Health Sci J 24:197-202.
15. **de Melker, H. E., F. G. A. Versteegh, J. F. P. Schellekens, P. F. M. Teunis, and M. Kretzschmar.** 2006. The incidence of Bordetella pertussis infections estimated in the population from a combination of serological surveys. Journal of Infection 53:106.
16. **Dethlefsen, L., M. McFall-Ngai, and D. A. Relman.** 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449:811-818.
17. **Diavatopoulos, D. A., C. A. Cummings, L. M. Schouls, M. M. Brinig, D. A. Relman, and F. R. Mooi.** 2005. Bordetella pertussis, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of B. bronchiseptica. PLoS Pathog 1:e45.
18. **Edwards, J. A., N. A. Groathouse, and S. Boitano.** 2005. Bordetella bronchiseptica adherence to cilia is mediated by multiple adhesin factors and blocked by surfactant protein A. Infect Immun 73:3618-3626.
19. **Gerlach, G., F. von Wintzingerode, B. Middendorf, and R. Gross.** 2001. Evolutionary trends in the genus Bordetella. Microbes Infect 3:61-72.
20. **Goodnow, R. A.** 1980. Biology of Bordetella bronchiseptica. Microbiol Rev 44:722-738.
21. **Gueirard, P., C. Weber, A. Le Coustumier, and N. Guiso.** 1995. Human Bordetella bronchiseptica infection related to contact with infected animals: persistence of bacteria in host. J Clin Microbiol 33:2002-2006.
22. **Harvill, E. T., P. A. Cotter, and J. F. Miller.** 1999. Pregenomic comparative analysis between bordetella bronchiseptica RB50 and Bordetella pertussis tohama I in murine models of respiratory tract infection. Infect Immun 67:6109-6118.
23. **Hijnen, M.** 2006. The Bordetella pertussis protein Pertactin: Role in Immunity and Immune Evasion. Eijkman Winkler Institute.
24. **Imaizumi, A., Y. Suzuki, S. Ono, H. Sato, and Y. Sato.** 1983. Heptakis(2,6-O-dimethyl)beta-cyclodextrin: a novel growth stimulant for Bordetella pertussis phase I. J Clin Microbiol 17:781-786.
25. **Kirimanjeswara, G. S., P. B. Mann, and E. T. Harvill.** 2003. Role of antibodies in immunity to Bordetella infections. Infect Immun 71:1719-1724.
26. **Kirimanjeswara, G. S., P. B. Mann, M. Pilione, M. J. Kennett, and E. T. Harvill.** 2005. The complex mechanism of antibody-mediated clearance of Bordetella from the lungs requires TLR4. J Immunol 175:7504-7511.

27. **Kuiken, T., E. C. Holmes, J. McCauley, G. F. Rimmelzwaan, C. S. Williams, and B. T. Grenfell.** 2006. Host species barriers to influenza virus infections. Science 312:394-397.
28. **Liese, J. G., C. Renner, S. Stojanov, B. H. Belohradsky, and G.** The Munich Vaccine Study. 2003. Clinical and epidemiological picture of B pertussis and B parapertussis infections after introduction of acellular pertussis vaccines. Arch Dis Child 88:684-687.
29. **Llombart, M., E. Chiner, and C. Senent.** 2006. [Necrotizing pneumonia due to Bordetella bronchiseptica in an immunocompetent woman]. Arch Bronconeumol 42:255-256.
30. **Lorenzo-Pajuelo, B., J. L. Villanueva, J. Rodriguez-Cuesta, N. Vergara-Irigaray, M. Bernabeu-Wittel, A. Garcia-Curiel, and G. Martinez de Tejada.** 2002. Cavitary pneumonia in an AIDS patient caused by an unusual Bordetella bronchiseptica variant producing reduced amounts of pertactin and other major antigens. J Clin Microbiol 40:3146-3154.
31. **Lysenko, E. S., A. J. Ratner, A. L. Nelson, and J. N. Weiser.** 2005. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. PLoS Pathog 1:e1.
32. **Maixnerova, M.** 2003. The 2001 serological survey in the Czech Republic--pertussis. Cent Eur J Public Health 11 Suppl:S17-22.
33. **Mattoo, S., and J. D. Cherry.** 2005. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin Microbiol Rev 18:326-382**.**
34. **Mira, A., R. Pushker, and F. Rodriguez-Valera. 2006.** The Neolithic revolution of bacterial genomes. Trends Microbiol 14:200-206.
35. **Parkhill, J., M. Sebaihia, A. Preston, L. D. Murphy, N. Thomson, D. E. Harris, M. T. Holden, C. M. Churcher, S. D. Bentley, K. L. Mungall, A. M. Cerdeno-Tarraga, L. Temple, K. James, B. Harris, M. A. Quail, M. Achtman, R. Atkin, S. Baker, D. Basham, N. Bason, I. Cherevach, T. Chillingworth, M. Collins, A. Cronin, P. Davis, J. Doggett, T. Feltwell, A. Goble, N. Hamlin, H. Hauser, S. Holroyd, K. Jagels, S. Leather, S. Moule, H. Norberczak, S. O'Neil, D. Ormond, C. Price, E. Rabbinowitsch, S. Rutter, M. Sanders, D. Saunders, K. Seeger, S. Sharp, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, L. Unwin, S. Whitehead, B. G. Barrell, and D. J. Maskell.** 2003. Comparative analysis of the genome sequences of Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Nat Genet 35:32-40.
36. **Pishko, E. J., G. S. Kirimanjeswara, M. R. Pilione, L. Gopinathan, M. J. Kennett, and E. T. Harvill.** 2004. Antibody-mediated bacterial clearance from the lower respiratory tract of mice requires complement component C3. Eur J Immunol 34:184-193.

37. **Poolman, J. T., and H. O. Hallander.** 2007. Acellular pertussis vaccines and the role of pertactin and fimbriae. Expert Rev Vaccines 6:47-56.
38. **Restif, O., and B. T. Grenfell. 2006.** Integrating life history and cross-immunity into the evolutionary dynamics of pathogens. Proc Biol Sci 273:409-416.
39. **Shapiro, S., D. O. Beenhouwer, M. Feldmesser, C. Taborda, M. C. Carroll, A. Casadevall, and M. D. Scharff.** 2002. Immunoglobulin G monoclonal antibodies to Cryptococcus neoformans protect mice deficient in complement component C3. Infect Immun 70:2598-2604.
40. **Stainer, D. W., and M. J. Scholte.** 1970. A simple chemically defined medium for the production of phase I Bordetella pertussis. J Gen Microbiol 63:211-220.
41. **Stibitz, S., and M. S. Yang. 1991.** Subcellular localization and immunological detection of proteins encoded by the vir locus of Bordetella pertussis. J Bacteriol 173:4288-4296.
42. **Tan, M. W. 2002.** Cross-species infections and their analysis. Annu Rev Microbiol 56:539-565.
43. **Taylor, L. H., S. M. Latham, and M. E. Woolhouse.** 2001. Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci 356:983-989.
44. **Tuomanen, E. I., J. Nedelman, J. O. Hendley, and E. L. Hewlett.** 1983. Species specificity of Bordetella adherence to human and animal ciliated respiratory epithelial cells. Infect Immun 42:692-695.
45. **van den Berg, B. M., S. David, H. Beekhuizen, F. R. Mooi, and R. van Furth.** 2000. Protection and humoral immune responses against Bordetella pertussis infection in mice immunized with acellular or cellular pertussis immunogens. Vaccine 19:1118-1128.
46. **van der Zee, A., F. Mooi, J. Van Embden, and J. Musser.** 1997. Molecular evolution and host adaptation of Bordetella spp.: phylogenetic analysis using multilocus enzyme electrophoresis and typing with three insertion sequences. J Bacteriol 179:6609-6617.
47. **von Koenig, C. H., A. Tacken, and H. Finger.** 1988. Use of supplemented Stainer-Scholte broth for the isolation of Bordetella pertussis from clinical material. J Clin Microbiol 26:2558-2560.
48. **Wolfe, D. N., G. S. Kirimanjeswara, E. M. Goebel, and E. T. Harvill.** 2007. Comparative role of immunoglobulin A in protective immunity against the Bordetellae. Infect Immun 75:4416-4422.
49. **Wolfe, D. N., G. S. Kirimanjeswara, and E. T. Harvill.** 2005. Clearance of Bordetella parapertussis from the lower respiratory tract requires humoral and cellular immunity. Infect Immun 73:6508-6513.
50. **Wolfe, N. D., C. P. Dunavan, and J. Diamond.** 2007. Origins of major human infectious diseases. Nature 447:279-283.

51. **Woodard, D. R., L. A. Cone, and K. Fostvedt.** 1995. Bordetella bronchiseptica infection in patients with AIDS. Clin Infect Dis 20:193-194.

52. **Woolfrey, B. F., and J. A. Moody.** 1991. Human infections associated with Bordetella bronchiseptica. Clin Microbiol Rev 4:243-255.

53. **Zhang, Y., S. Pacheco, C. L. Acuna, K. C. Switzer, Y. Wang, X. Gilmore, G. R. Harriman, and I. N. Mbawuike.** 2002. Immunoglobulin A-deficient mice exhibit altered T helper 1-type immune responses but retain mucosal immunity to influenza virus. Immunology 105:286-294.

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Chapter 3: O-antigen is a Critical Antigen for the Development of a Protective Immune Response to *Bordetella parapertussis*

Abstract:

Whooping cough is re-emerging in vaccinated populations. This resurgence could be due in part to the use of vaccines that are only efficious against one of the causative agents, *Bordetella pertussis*, but not the other, *B. parapertussis*. Identifying and including the protective antigens of *B. parapertussis* in the vaccine formula could help decrease the overall incidence of whooping cough disease. As O-antigen, a known protective antigen for other pathogenic gram negative bacteria, is expressed by *B. parapertussis* but not *B. pertussis*; we examined this factor as a potential protective antigen. Using a mouse model of infection, we show that immunization with wild type *B. parapertussis* conferred efficient protection against a subsequent *B. parapertussis* challenge, but immunization with the isogenic mutant lacking O-antigen *B. parapertussisΔwbm* did not. Inoculation with *B. parapertussis* induced a strong antibody response to O-antigen. In addition, the passive transfer of antibodies raised against *B. parapertussis*, but not *B. parapertussisΔwbm*, reduced bacterial load in the respiratory tract. The addition of 10μg of purified *B. parapertussis* lipopolysaccharide, which contains the O-antigen, drastically improved the efficacy of the acellular vaccine, Adacel®, against this pathogen. Although current whooping cough vaccines are ineffective against *B. parapertussis*, these data suggest that O-antigen is a critical protective antigen of *B. parapertussis* and the inclusion of a conjugated form of this antigen could improve whooping cough vaccine efficiency.

Introduction:

B. pertussis and *B. parapertussis* are the two causative agents of whooping cough, causing approximately 50 million cases and 300,000 deaths annually worldwide (20). Whooping cough has recently been classified as a re-emerging disease by the CDC (5); but the ratio of *B. pertussis*-caused versus *B. parapertussis*-caused cases in this resurgence is unknown (37). It is known, however, that the resurgence of whooping cough roughly correlates with the introduction of acellular pertussis vaccines (5). These vaccines contain only *B. pertussis*-derived antigens, some of which are also expressed by *B. parapertussis,* such as pertactin and filamentous hemagglutinin, but do not contain *Bordetella* lipopolysaccharide (LPS) (20).

Although *B. pertussis* and *B. parapertussis* are very closely related (7, 24, 35), they differ in the structure of their LPS (1, 2, 28, 29, 34). *B. pertussis* produces a lipooligosaccharide containing lipid A and a branched-chain core oligosaccharide with a complex trisaccharide modification, but completely lacks O-antigen (28, 34). *B. parapertussis* produces an LPS molecule that has a distinct, truncated lipid A and a core oligosaccharide lacking the trisaccharide modification, but includes an O-antigen (28, 29). In addition to conferring serum resistance, O-antigen enables *B. parapertussis* to avoid *B. pertussis*-induced immunity by inhibiting antibody binding to the surface of the bacteria (10, 39). Thus, O-antigen facilitates *B. parapertussis* infection in hosts already immune to *B. pertussis* by inhibiting the function of a *B. pertussis*-induced adaptive immune response (3, 31, 39). As *B. pertussis* does not express an O-antigen, immunization with this pathogen does not induce an immune response to O-antigen. Since O-antigen has been shown to be a protective antigen of other bacterial pathogens (15, 25), it may also be a critical antigen for the development of a protective immune response to *B. parapertussis*. Therefore, we hypothesize that the lack of an immune response to O-antigen may contribute to the ability of *B. parapertussis* to evade current whooping cough vaccination strategies. However, the importance of this antigen during the development of an adaptive immune response has not been fully addressed.

In order to assess the role of O-antigen during the generation of an adaptive immune response to *B. parapertussis*, mice were infected or vaccinated with *B. parapertussis* or an isogenic mutant lacking O-antigen. Animals immunized with *B. parapertussis*, but not *B. parapertussis* lacking O-antigen (*Δwbm*), were protected against subsequent challenge with *B. parapertussis*. Mice immunized with *B. parapertussisΔwbm* were also deficient in the production of *B. parapertussis*-specific antibodies, and these antibodies were less effective at reducing *B. parapertussis* colonization upon passive transfer compared to antibodies raised against *B. parapertussis*. In addition, the inclusion of *B. parapertussis* LPS rendered the acellular *B. pertussis* vaccine Adacel® effective against *B. parapertussis* challenge. This indicates that O-antigen is an important protective antigen of *B. parapertussis* and that

an effective immune response must target this antigen for protection against this bacterium.

Materials and Methods:

Bacterial strains and growth. *B. pertussis* strain 536, *B. parapertussis* strains CN2591 and the isogenic mutant lacking O-antigen, CN2591*Δwbm*, have been described previously (28). Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% defibrinated sheep blood (Hema Resources) and 20μg/ml streptomycin (Sigma-Aldrich). Liquid cultures were grown overnight on a roller drum at 37°C to mid-log phase in Stainer-Scholte broth (32, 36).

Animal experiments. C57BL/6 mice were obtained from Jackson Laboratories. $C3^{-/-}$ mice were kind gifts from Dr. Rick Wetsel and have been described elsewhere (6). All mice were bred in our *Bordetella*-free, specific pathogen-free breeding rooms at The Pennsylvania State University. 4-6 week old mice were sedated with 5% isoflourane (Abbott Laboratory) in oxygen and inoculated by pipetting 50μl of phosphate balanced saline (PBS, Omnipur) containing 5×10^5 CFU of bacteria onto the external nares (13). This method reliably distributes the bacteria throughout the respiratory tract (11). For challenge experiments, mice were treated with gentamicin via drinking water (10mg/ml) for 7 days starting on day 21 post-inoculation (40). On day 30 post-inoculation, mice were challenged with 5×10^5 CFU of the indicated bacteria and dissected 3 days post-challenge (40). For passive transfer of immune serum, 200μL of sera from naïve or convalescent $C3^{-/-}$ mice (collected day 28 post-inoculation) were intraperitoneally (i.p.) injected at the time of inoculation (14, 27). For vaccination, mice were i.p. injected with 1×10^8 CFU of heatkilled CN2591 or CN2591*Δwbm* in 200μL of PBS with Imject Alum (Pierce) on days 0 and 14. For acellular *B. pertussis* vaccinations, mice were given a 1/5 human dose of Adacel® (Sanofi Pastuer) with Imject Alum with or without 10μg of purified *B. parapertussis* LPS (33) on days 0 and 14. Vaccinated mice were challenged with the indicated bacteria on day 28. Mice were sacrificed via CO_2 inhalation and the lungs, tracheae, and nasal cavities were excised. Tissues were homogenized in PBS, serial diluted and plated onto Bordet-Gengou agar, and colonies were counted after incubation at 37°C for 3-4 days (19). All protocols were reviewed and approved by the university IACUC and all animals were handled in accordance with institutional guidelines.

Splenocyte re-stimulations. Spleens were taken from C57BL/6 mice immunized with CN2591 or CN2591*Δwbm* on day 28 post-inoculation. Splenocytes were isolated as previously described (19, 26). In brief, spleens were homogenized and red blood cells were lysed with 0.84% ammonium chloride treatment. 2×10^6 cells were re-suspended in Dulbecco's modified Eagle cell culture medium (DMEM) (HyClone) supplemented with 10% fetal calf serum (HyClone), 1 mM sodium pyruvate (HyClone), and 100 μg/ml penicillin and streptomycin (HyClone) and placed into each well of a 96-well tissue culture plate. Splenocytes were stimulated with either medium alone or medium

containing 10^7 heat-killed CN2591 or CN2591Δ*wbm* (26). After three days, the supernatants were collected and analyzed for IFN-γ and IL-10 production via sandwich ELISA as per the manufacturers' instructions (R&D Systems). All samples were run in triplicate and independently repeated.

Enzyme-linked immunosorbent assays (ELISAs). Antibody titer was determined as previously described (19, 39). In brief, exponential phase, live CN2591 or CN2591Δ*wbm* were coated onto 96-well plates and a 1:50 dilution of the indicated serum samples was serially diluted across the plate and incubated for 2h at 37°C. Plates were probed with 1:4000 dilution of goat anti-mouse Ig or IgG HRP-conjugated antibodies (Southern Biotech) for 1 hour and washed prior to visualization with Azino-bis (3-ethylbenz-thiazoline-6-sulfnic acid) in phospho-citrate buffer at an absorbance of 405 nm. Titers were determined via the endpoint method.

Western Blot Analysis. Lysates containing 1×10^7 CFU of heat-killed CN2591 or CN2591*Δwbm* as indicated were run on 10% SDS-PAGE gels in denaturing conditions. PVDF membranes (Millipore) were probed with either naïve serum or serum from CN2591 or CN2591*Δwbm*-inoculated mice as indicated at a 1:50 dilution overnight. Goat anti-mouse Ig (H+L) HRP-conjugated antibodies (Southern Biotech) were used at a dilution of 1:10,000 as the detector antibody (39, 40). The membrane was visualized with ECL Western Blotting Detection Reagent (Pierce Biotechnology).

Statistical Analysis. The mean +/- standard error was determined for all appropriate data. Two-tailed, unpaired Student's T-tests were used to determine statistical significance between groups. All experiments were performed at least twice with similar results.

Results:

O-antigen enables *B. parapertussis* to colonize *B. pertussis*-immune hosts. The coexistence of *B. pertussis* and *B. parapertussis* in humans led us to assess the ability of these pathogens to induce cross-protective immunity. Mice were left uninfected or inoculated with *B. pertussis* or *B. parapertussis* and 28 days later challenged with wild-type or O-antigen-deficient *B. parapertussis* and then dissected for CFU 3 days post-secondary challenge. *B. parapertussis* efficiently colonized naïve animals, with $10^{6.2}$, 10^{6} and $10^{6.4}$ CFU in the nasal cavity, trachea and lungs, respectively. Compared to naïve mice, *B. parapertussis* numbers were only 5-fold lower in the nasal cavities and approximately 20-fold lower in the tracheae and lungs of *B. pertussis*-immunized animals. However, the bacterial numbers were approximately 100-fold lower in the nasal cavities and 100,000-fold lower in the tracheae and lungs of *B. parapertussis*-convalescent mice than in those of naive mice. The O-antigen-deficient strain of *B. parapertussis* was present at approximately 10^{6}, 10^{6}, and $10^{5.2}$ CFU in the nasal cavity, trachea, and lungs of naive mice, respectively, but bacterial numbers were approximately 10-fold lower in the nasal cavities and 100,000-fold lower in the tracheae and lungs of *B. pertussis*-convalescent or *B. parapertussis*-convalescent mice (Figure 3.1). Thus, O antigen is required for *B. parapertussis* to evade *B. pertussis*-induced immunity and colonize *B. pertussis*-immune hosts. Because *B. pertussis* does not express an O-antigen, and O-antigen allows *B. parapertussis* to evade *B. pertussis*-induced immunity, the role of O-antigen during the generation of an effective adaptive immune response to *B. parapertussis* was examined.

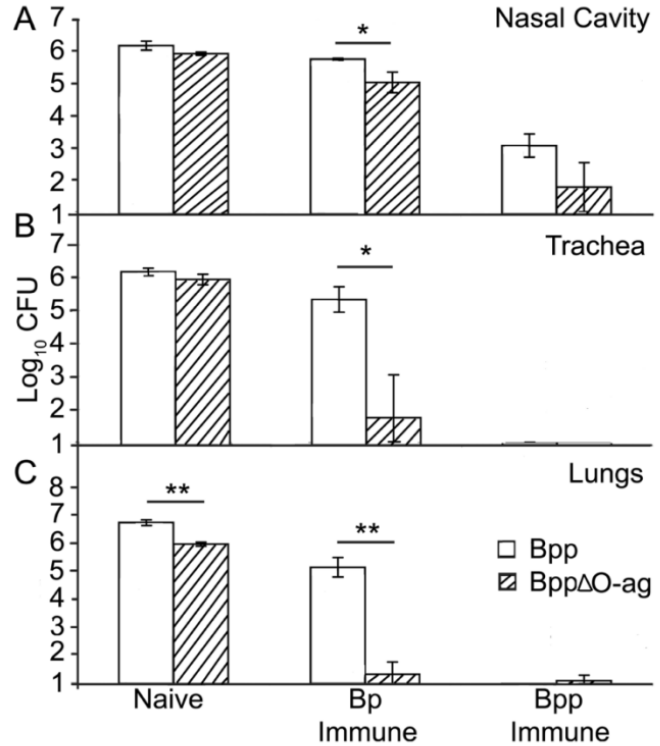

Figure 3.1: Ability of the O antigen-deficient strain of *B. parapertussis* to colonize *B. pertussis*-immune hosts. Groups of four C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis* (Bp) or *B. parapertussis* (Bpp). Immunized and naive mice were challenged 28 days later with 5×10^5 CFU of Bpp (white bars) or the O antigen-deficient strain of *B. parapertussis* (BppΔO-ag, hatched bars). Mice were sacrificed 3 days post-secondary inoculation for the quantification of bacterial numbers in the nasal cavity (A), trachea (B), and lungs (C). All values are expressed as Log_{10} mean +/- SD. One asterisk represents P-values < 0.05, two asterisks represent P-values < 0.01.

O-antigen is required for efficient generation of protective immunity to *B. parapertussis.* To test the hypothesis that generation of an effective immune response to *B. parapertussis* requires the presence of O-antigen; mice were inoculated with either *B. parapertussis* or *B. parapertussisΔwbm*. Three weeks later, mice were gentimicin-treated to clear remnant bacteria (40), and then challenged four weeks post-inoculation with either *B. parapertussis* or *B. parapertussisΔwbm*. Mice were dissected 3 days post-challenge for bacterial enumeration. Naïve animals challenged with *B. parapertussis* showed $10^{6.3}$ CFU in the nasal cavity, $10^{6.1}$ CFU in the trachea and $10^{6.8}$ CFU in the lungs (Figure 3.2). Prior immunization with *B. parapertussis* caused a 2000 fold, 100,000 fold and 500,000 fold decrease in bacterial numbers in the nasal cavity, trachea and lungs, respectively, when compared to naïve animals. However, prior immunization with *B. parapertussisΔwbm* was less effective, causing a 1000 fold, 100,000 fold and 100 fold decrease in bacterial number in the nasal cavity, trachea and lungs, respectively, when compared to naïve animals (Figure 3.2). Prior immunization with either *B. parapertussis* or *B. parapertussisΔwbm* conferred similar protection against subsequent *B. parapertussisΔwbm* challenge in the lower respiratory tract. The difference in protection in the lower respiratory tract indicates that O-antigen may be important for the development of an effective adaptive immune response against *B. parapertussis*.

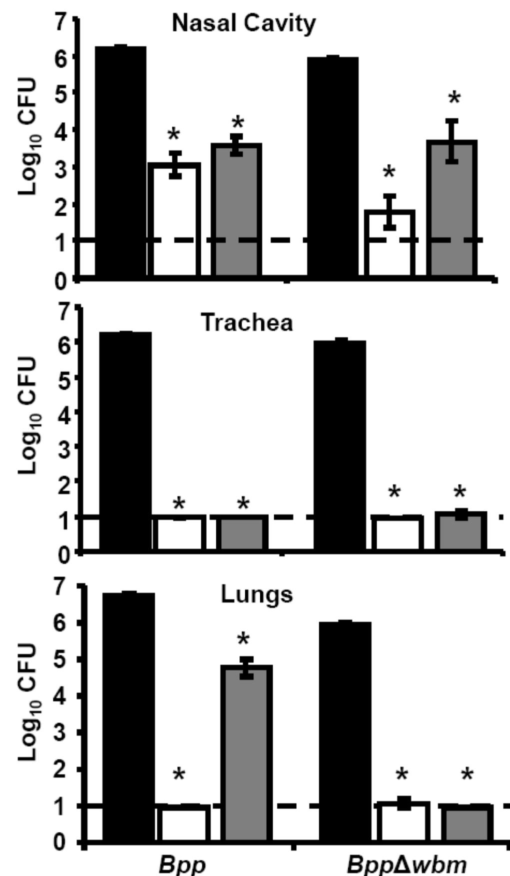

Figure 3.2: O-antigen is required for efficient generation of protective immunity to *B. parapertussis*. Groups of four 4 – 6 week-old C57BL/6 mice were inoculated with *B. parapertussis* (white bars), *B. parapertussisΔwbm* (grey bars) and allowed to convalesce for 28 days. Naïve (black bars) and immunized mice were challenged with the indicated bacteria and dissected on day 3 post-challenge. The number of CFU recovered from the nasal cavity, trachea and lungs at day 3 post-challenge is expressed as the $\log_{10}$ mean ± the standard error. * indicates $P \leq 0.05$. The limit of detection is indicated by the dashed line.

Effective vaccine induced immunity requires a response against O-antigen. *B. parapertussisΔwbm* is known to colonize at a lower level than *B. parapertussis* in the presence of complement (10). To rule out the possibility that the defect in colonization of *B. parapertussisΔwbm* contributes to the decreased immune response seen in these mice, mice were vaccinated with 1 x 10^8 CFU of heatkilled *B. parapertussis* or *B. parapertussisΔwbm* with a commercially available adjuvant. Mice sham-vaccinated with adjuvant and PBS harbored $10^{6.4}$, $10^{5.8}$ and $10^{6.8}$ CFU in the nasal cavity, trachea and lungs upon challenge with *B. parapertussis* (Figure 3.3). Mice vaccinated with *B. parapertussis* effectively decreased a secondary challenge of *B. parapertussis* with $10^{5.6}$, $10^{2.0}$ and $10^{2.7}$ CFU in the nasal cavity, trachea and lungs, representing a 10 fold, 6000 fold and 10,000 fold reduction in bacterial load, respectively (Figure 3.3). Similar to inoculation, mice vaccinated with *B. parapertussisΔwbm* were susceptible to *B. parapertussis* challenge with $10^{6.1}$, $10^{3.2}$, and $10^{4.9}$, in the nasal cavity, trachea and lungs, representing an insignificant decrease, a 300 fold and a 90 fold reduction in bacterial load, respectively. This indicates that the decreased protection observed in *B. parapertussisΔwbm*-immunized mice is not caused by decreased colonization by the O-antigen mutant strain and further strengthens the conclusion that O-antigen is required for the generation of an effective adaptive immune response against *B. parapertussis.*

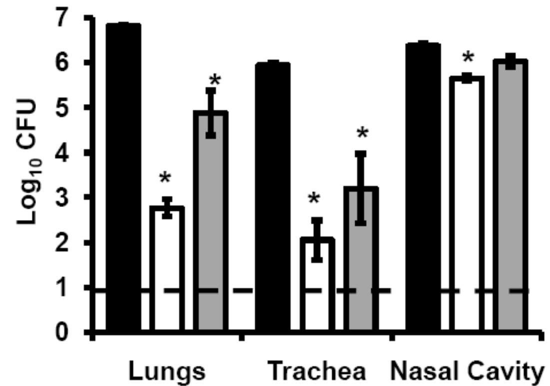

Figure 3.3: Effective vaccine-induced immunity requires a response against O-antigen. Groups of four 4 – 6 week-old C57BL/6 mice were vaccinated with adjuvant only (black bars), *B. parapertussis* with adjuvant (white bars), or *B. parapertussisΔwbm* with adjuvant (grey bars) and challenged with *B. parapertussis*. The number of CFU recovered from the nasal cavity, trachea and lungs at day 3 post-challenge is expressed as the $\log_{10}$ mean ± the standard error. * indicates $P \leq 0.05$. The limit of detection is indicated by the dashed line.

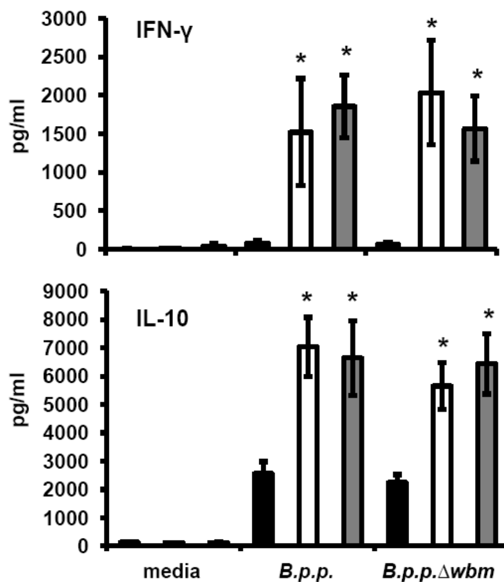

Figure 3.4: O-antigen is not required for the development of IFN-γ or IL-10 producing T cells in response to *B. parapertussis*. Splenocytes from groups of four 4 – 6 week-old C57BL/6 mice were vaccinated with adjuvant only (black bars), *B. parapertussis* with adjuvant (white bars), or *B. parapertussisΔwbm* with adjuvant (grey bars) were stimulated with the indicated bacteria for 3 days, and the resulting IFN-γ and IL-10 production was assessed. * indicates $P \leq 0.05$.

O-Antigen is not required for development of a T cell response to *B. parapertussis*. To investigate whether O-antigen was involved in the generation of a productive T cell response, mice were vaccinated with *B. parapertussis* or *B.*

parapertussisΔwbm on days 0 and 14. On day 28, the spleens were excised and stimulated with either heatkilled *B. parapertussis* or *B. parapertussisΔwbm*. There was no significant difference in either Interferon (IFN) – γ or Interleukin (IL) – 10 production by splenocytes stimulated with either *B. parapertussis* or *B. parapertussisΔwbm* from *B. parapertussis* or *B. parapertussisΔwbm* vaccinated mice (Figure 3.4). Similar results were obtained from wildtype mice and $C3^{-/-}$ mice infected with *B. parapertussis* or *B. parapertussisΔwbm* (data not shown). These data indicate that the presence of O-antigen is not required for the generation of a T cell cytokine response to *B. parapertussis*.

O-Antigen is required for efficient antibody generation to *B. parapertussis* antigens. As O-antigen is required for the generation of an effective immune response to *B. parapertussis*, but is not required for efficient T cell generation, we sought to determine if the antibody response generated in response to *B. parapertussis* was similar to that of the O-antigen mutant. Serum raised against *B. parapertussis* contained a *B. parapertussis*-specific titer of $10^{5.3}$ (Figure 3.5A). Serum raised against the O-antigen mutant of *B. parapertussis* contained significantly less *B. parapertussis*-specific antibodies, with a titer of $10^{4.9}$ (Figure 3.5A). In addition, *B. parapertussis*-

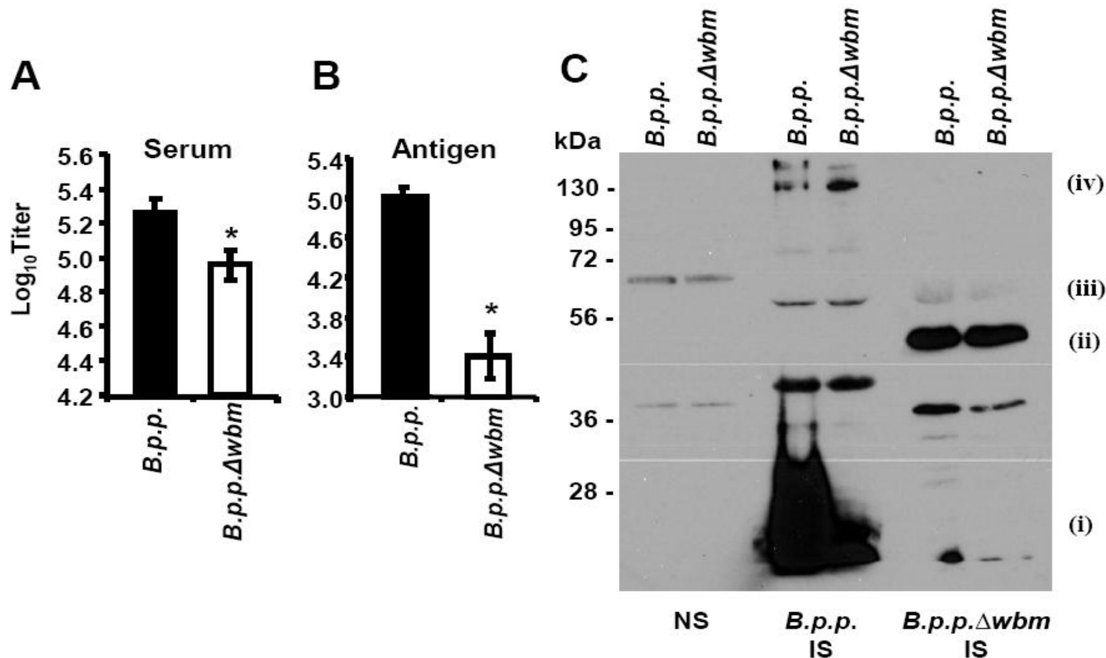

Figure 3.5: O-antigen is required for the production of a robust α-*B. parapertussis* antibody response. Serum antibody titer from groups of 4 C57BL/6 mice (A) inoculated with *B. parapertussis* (black bar) or *B. parapertussisΔwbm* (white bar) was determined via *B. parapertussis*-specific ELISA. Serum antibody titer from groups of 4 C57BL/6 mice (B) vaccinated with *B. parapertussis* was determined via a *B. parapertussis*-specific (black bar) or *B. parapertussisΔwbm*-specific (white bar) ELISA. * indicates $P \leq 0.05$. (C) 1×10^7 CFU of *B. parapertussis* (*B.p.p.*) or *B. parapertussisΔwbm* (*B.p.p.Δwbm*) lysates were loaded per well and separated via SDS-PAGE. Proteins were transferred to PVDF membrane, and then probed with either naïve serum (NS), *B. parapertussis*-induced serum (*B.p.p.* IS) or *B. parapertussisΔwbm*-induced serum (*B.p.p.Δwbm* IS), as indicated. The membrane was visualized via an ECL detection kit.

induced serum had significantly less recognition of the O-antigen mutant as compared to the wild type bacteria (Figure 3.5B), while *B. parapertussisΔwbm*-induced serum had similar titers when either the wild type or O-antigen mutant bacteria were probed (data not shown). These data suggest that vaccination with *B. parapertussis* induces a robust antibody response against O-antigen.

To determine if decreased antibody titer correlated to different antigen recognition patterns, lysates of *B. parapertussis* and *B. parapertussisΔwbm* were probed with a 1:250 dilution of naïve, or 1:500 dilution of *B. parapertussis*-induced, or *B. parapertussisΔwbm*-induced serum (Figure 3.5C). Naïve serum had little recognition of either lysate. *B. parapertussis*-induced serum recognized several bands. The strongest band was O-antigen (band i), indicating that this antigen is strongly recognized by the immune system. Several higher molecular weight bands were also observed, and, by size, were suspected to be filamentous hemagglutinin (band iv) and pertactin (band iii), two factors known to be antigenic in the bordetellae (20). Interestingly, *B. papapertussisΔwbm*-induced serum did not recognize the largest of the bands, band (iv), but did have weak recognition of band (iii) and had strong recognition of an additional antigen (band ii) not seen in *B. parapertussis*-induced serum. As expected, no recognition of O-antigen (band i) was seen in *B. parapertussisΔwbm*-induced serum. Together, these data indicate that infection with *B. parapertussis* induces a measurably stronger antibody response which recognizes distinct antigens.

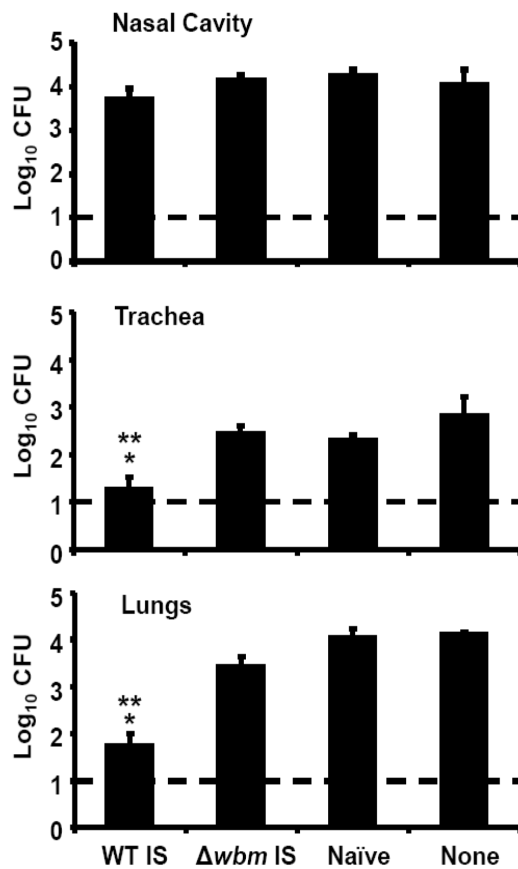

Figure 3.6: Antibodies to O-antigen are required for efficient antibody-mediated clearance of *B. parapertussis*. Groups of four 4 – 6 week-old C57BL/6 mice were inoculated with *B. parapertussis* and i.p. injected with the indicated serum. Bacterial load in the nasal cavity, trachea, and lungs 14 days post-inoculation is expressed as the $\log_{10}$ mean ± the standard error. * indicates $P \leq 0.05$ between naïve and *B. parapertussis*-induced serum. ** indicates $P \leq 0.05$ between *B. parapertussisΔwbm*-induced serum and *B. parapertussis*-induced serum. The limit of detection is indicated by the dashed line.

Antibodies to O-antigen are required for efficient antibody mediated clearance of *B. parapertussis*. To determine if the antibodies raised against *B. parapertussis* were more efficient than antibodies raised against *B. parapertussisΔwbm* at decreasing *B. parapertussis* colonization upon i.p. injection, mice were i.p. injected with immune or naive sera and immediately challenged with *B. parapertussis*. Because *B. parapertussisΔwbm* is known to have

decreased colonization in complement-sufficient mice (10), all sera used in this experiment were generated in complement-deficient mice. By day 14 post-inoculation, naïve serum had no effect on bacterial load in the nasal cavity, trachea, or lungs with $10^{4.2}$, $10^{2.5}$ and 10^{4} CFU, respectively (Figure 3.6). As seen in previous studies (14, 39), *B. parapertussis*-induced serum decreased the bacterial load in the trachea and lungs to $10^{1.1}$ and $10^{1.6}$ CFU, respectively. However, *B. parapertussis*Δ*wbm*-induced serum failed to reduce *B. parapertussis* colonization in the respiratory tract, with $10^{2.6}$ and $10^{3.6}$ CFU in the trachea and lungs, respectively (Figure 3.6), further supporting our hypothesis that an immune response to O-antigen is important to protection against *B. parapertussis.*

Adacel® with purified *B. parapertussis* LPS is sufficient to induce protection against *B. parapertussis* challenge. To determine if the addition of *B. parapertussis* LPS containing O-antigen to current whooping cough vaccines would render the vaccines protective against a *B. parapertussis* challenge, mice were vaccinated with adjuvant only, a 1/5 human dose of Adacel®, an acellular pertussis vaccine which contains the *B. pertussis*-derived antigens of pertussis toxin, filamentous hemagglutinin, pertactin, and fimbrea 2 and 3, or a 1/5 human dose of Adacel® with 10µg of purified *B. parapertussis* LPS. Vaccination with Adacel® had no significant effect on *B. parapertussis* load throughout the respiratory tract (Figure 3.7). In contrast, the addition of *B. parapertussis* LPS to Adacel® caused a significant decrease in bacterial load throughout the respiratory tract with a 1000 fold decrease in bacterial load in the lungs and trachea and a 10 fold decrease in the nasal cavity of immunized mice. This indicates that an immune response to *B. parapertussis* LPS is important to the efficient control and reduction of this bacterium.

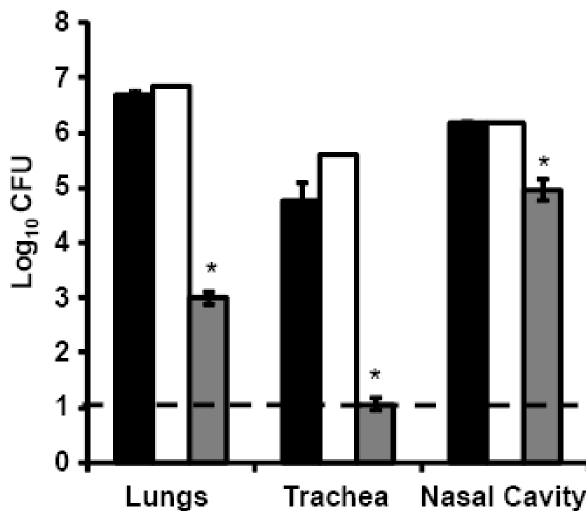

Figure 3.7: Addition of purified *B. parapertussis* LPS to an acellular *B. pertussis* vaccine confers protection against *B. parapertussis* challenge. Groups of four 4 – 6 week-old C57BL/6 mice were vaccinated with either PBS (black bars), 1/5 human dose of Adacel® and adjuvant (white bars), or 1/5 of the human dose of Adacel® and adjuvant with 10µg of *B. parapertussis* LPS (grey bars). Mice were challenged with *B. parapertussis* and dissected on day 3 post-challenge. The number of CFU recovered from the nasal cavity, trachea and lungs at day 3 post-challenge is expressed as the log_{10} mean ± the standard error. * indicates P ≤ 0.05. The limit of detection is indicated by the dashed line.

Discussion:

The most variable portion of LPS, the O-antigen, is critical for the generation of an effective adaptive immune response to *B. parapertussis*. Prior infection or vaccination with B*. pertussis* or an O-antigen deficient strain of *B.* parapertussis failed to induce protective immunity against a wild type strain of *B. parapertussis* (Figure 3.1, 3.2 and 3.3). In contrast, immunization with wild type *B. parapertussis* does induce protective immunity against both the wild type and O-antigen mutant. Further investigation showed that vaccination with *B. parapertussisΔwbm* induced similar T-cell responses as that of *B. parapertussis* vaccination (Figure 3.4) indicating that the defect in protection was not due to inefficient T cell cytokine production. Interestingly, western blot analysis revealed that *B. parapertussis*-induced antibodies recognized distinct bands, of which the strongest was O-antigen (Figure 3.5). Antibodies in serum induced by *B. parapertussisΔwbm* failed to recognize O-antigen and had lower overall *B. parapertussis*-specific titers when compared to serum raised against *B. parapertussis*. Further supporting the hypothesis that *B. parapertussisΔwbm*-induced antibodies were insufficient to protect against a *B. parapertussis* challenge, antibodies raised against *B. parapertussis*, but not *B. parapertussisΔwbm*, reduced *B. parapertussis* colonization upon passive transfer (Figure 3.6). Taken together, these data suggest that O-antigen is required for the generation of an effective, protective antibody response against *B. parapertussis*.

The incidence of whooping cough has increased over the past 20 years, despite the maintenance of excellent vaccine coverage (5). This could be due, at least in part, because these vaccines are ineffective against *B. parapertussis*-induced disease (8, 12, 16). This inefficacy is either the result of the vaccine antigens not being cross-reactive with *B. parapertussis* antigens or the vaccines not containing the appropriate protective antigens. Interestingly, the addition of 10 ug of purified *B. parapertussis* LPS to the acellular pertussis vaccine, Adacel®, induced protection (Figure 3.7), indicating that supplementing this vaccine with a protective antigen of *B. parapertussis* renders the vaccine effective against this pathogen.

Currently, lack of reporting of *B. parapertussis* infections and difficulties in differentiating between it and *B. pertussis* have resulted in an unclear picture of the prevalence of *B. parapertussis*. Recent studies have suggested that *B. parapertussis* is more common as the causative agent of whooping cough in pertussis vaccinated individuals compared to unvaccinated individuals (16-18), potentially due to a vaccine-mediated selective advantage for this pathogen relative to *B. pertussis*. Determining the protective antigens of *B. parapertussis* may allow the development of a whooping cough vaccine that is effective against both causative agents and may aid in decreasing the incidence of this disease.

Prior studies using *B. parapertussisΔwbm* have shown that this bacterium is deficient in colonization of wild type mice due to increased susceptibility to complement-

mediated control and killing (4, 10). To address the concern that the lack of protection conferred by *B. parapertussisΔwbm* could be a result of decreased colonization and therefore decreased immune stimulation, mice were vaccinated (Figure 4.2) with 1 x 10^8 CFU of heat-killed *B. parapertussis* or *B. parapertussisΔwbm* in a commercial adjuvant. This method removes any difference in immune stimulation due to colonization levels during infection. In addition, because *B. parapertussisΔwbm* is not defective in colonization of mice lacking complement , all sera used in adoptive transfers (Figure 4.5) were generated in $C3^{-/-}$ mice which are deficient in the complement cascade (6, 10, 38), thereby removing the difference in CFU load as a factor in antibody production.

Despite being a protective antigen of *B. parapertussis*, there has not been any observed variability of O-antigen among clinical isolates (29, 34, 35). This suggests it must perform some critical function. In addition to its role in evading complement mediated killing (10), our lab has shown that O-antigen allows *B. parapertussis* to avoid *B. pertussis*-induced antibody responses (39). Therefore, maintenance of O-antigen could be advantageous to *B. parapertussis* as greater than 90% of a human population is seropositive for *B. pertussis* (9, 17, 18). Additionally, *B. parapertussis* is thought to have evolved in a population in which *B. pertussis* was already endemic (3, 31) and O-antigen may have contributed to this process by allowing *B. parapertussis* to avoid *B. pertussis* mediated-immunity (30, 31).

Current acellular pertussis vaccines contain some combination of pertussis toxin, which is *B. pertussis*-specific (22, 23), and four antigens that are shared between both *B. pertussis* and *B. parapertussis*; filamentous hemagglutinin, pertactin, and fimbrae 2 and 3 (20). Our results suggest that the addition of some conjugate form of *B. parapertussis* O-antigen to these vaccines could help reduce the incidence of this disease. The addition of LPS components to an acellular vaccine would have several drawbacks including public opinion, difficulty in acquiring FDA approval and increased reactigenicity. However, it is possible to purify the O-antigen portion of the LPS, thereby removing the TLR-4 antagonist, Lipid A, which is attributed most of the pro-inflammatory stimulation associated with LPS (21). Alternatively, other as of yet unidentified protein antigens of *B. parapertussis* may prove to be protective and could be added to acellular whooping cough vaccines. In light of the fact that *B. parapertussis* may cause more morbidity in vaccinated individuals (16), relative to unvaccinated individuals, a vaccine that protects against both causative agents of whooping cough could significantly decrease the incidence of whooping cough in vaccinated populations.

References:

1. **Allen, A., and D. Maskell.** 1996. The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in Bordetella pertussis. Mol Microbiol **19:**37-52.
2. **Allen, A. G., R. M. Thomas, J. T. Cadisch, and D. J. Maskell.** 1998. Molecular and functional analysis of the lipopolysaccharide biosynthesis locus wlb from Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Molecular Microbiology **29:**27-38.
3. **Bjornstad, O. N., and E. T. Harvill.** 2005. Evolution and emergence of Bordetella in humans. Trends Microbiol **13:**355-359.
4. **Burns, V. C., E. J. Pishko, A. Preston, D. J. Maskell, and E. T. Harvill.** 2003. Role of Bordetella O Antigen in Respiratory Tract Infection. Infect. Immun. **71:**86-94.
5. **CDC.** 2002. Pertussis--United States, 1997-2000. JAMA **287:**977-979.
6. **Circolo, A., G. Garnier, W. Fukuda, X. Wang, T. Hidvegi, A. J. Szalai, D. E. Briles, J. E. Volanakis, R. A. Wetsel, and H. R. Colten.** 1999. Genetic disruption of the murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA. Immunopharmacology **42:**135-149.
7. **Cummings, C. A., M. M. Brinig, P. W. Lepp, S. van de Pas, and D. A. Relman.** 2004. Bordetella species are distinguished by patterns of substantial gene loss and host adaptation. J Bacteriol **186:**1484-1492.
8. **David, S., R. van Furth, and F. R. Mooi.** 2004. Efficacies of whole cell and acellular pertussis vaccines against Bordetella parapertussis in a mouse model. Vaccine **22:**1892.
9. **de Melker, H. E., J. F. Schellekens, S. E. Neppelenbroek, F. R. Mooi, H. C. Rumke, and M. A. Conyn-van Spaendonck.** 2000. Reemergence of pertussis in the highly vaccinated population of the Netherlands: observations on surveillance data. Emerg Infect Dis **6:**348-357.
10. **Goebel, E. M., D. N. Wolfe, K. Elder, S. Stibitz, and E. T. Harvill.** 2008. O antigen protects Bordetella parapertussis from complement. Infect Immun **76:**1774-1780.
11. **Harvill, E. T., A. Preston, P. A. Cotter, A. G. Allen, D. J. Maskell, and J. F. Miller.** 2000. Multiple Roles for Bordetella Lipopolysaccharide Molecules during Respiratory Tract Infection. Infect. Immun. **68:**6720-6728.
12. **Heininger, U., K. Stehr, S. Schmitt-Grohe, C. Lorenz, R. Rost, P. D. Christenson, M. Uberall, and J. D. Cherry.** 1994. Clinical characteristics of illness caused by Bordetella parapertussis compared with illness caused by Bordetella pertussis. Pediatr Infect Dis J **13:**306-309.

13. **Kirimanjeswara, G. S., L. M. Agosto, M. J. Kennett, O. N. Bjornstad, and E. T. Harvill.** 2005. Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of Bordetella pertussis. J Clin Invest **115:**3594-3601.
14. **Kirimanjeswara, G. S., P. B. Mann, and E. T. Harvill.** 2003. Role of antibodies in immunity to Bordetella infections. Infect Immun **71:**1719-1724.
15. **Li, J., C. Ryder, M. Mandal, F. Ahmed, P. Azadi, D. S. Snyder, R. D. Pechous, T. Zahrt, and T. J. Inzana.** 2007. Attenuation and protective efficacy of an O-antigen-deficient mutant of Francisella tularensis LVS. Microbiology **153:**3141-3153.
16. **Liese, J. G., C. Renner, S. Stojanov, B. H. Belohradsky, and G. The Munich Vaccine Study.** 2003. Clinical and epidemiological picture of B pertussis and B parapertussis infections after introduction of acellular pertussis vaccines. Arch Dis Child **88:**684-687.
17. **Maixnerova, M.** 2003. The 2001 serological survey in the Czech Republic--parapertussis. Cent Eur J Public Health **11 Suppl:**S23-24.
18. **Maixnerova, M.** 2003. The 2001 serological survey in the Czech Republic--pertussis. Cent Eur J Public Health **11 Suppl:**S17-22.
19. **Mann, P., E. Goebel, J. Barbarich, M. Pilione, M. Kennett, and E. Harvill.** 2007. Use of a Genetically Defined Double Mutant Strain of Bordetella bronchiseptica Lacking Adenylate Cyclase and Type III Secretion as a Live Vaccine. Infect. Immun. **75:**3665-3672.
20. **Mattoo, S., and J. D. Cherry.** 2005. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin Microbiol Rev **18:**326-382.
21. **Munford, R. S., and A. W. Varley.** 2006. Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria. PLoS Pathog **2:**e67.
22. **Nicosia, A., A. Bartoloni, M. Perugini, and R. Rappuoli.** 1987. Expression and immunological properties of the five subunits of pertussis toxin. Infect Immun **55:**963-967.
23. **Nicosia, A., and R. Rappuoli.** 1987. Promoter of the pertussis toxin operon and production of pertussis toxin. J Bacteriol **169:**2843-2846.
24. **Parkhill, J., M. Sebaihia, A. Preston, L. D. Murphy, N. Thomson, D. E. Harris, M. T. Holden, C. M. Churcher, S. D. Bentley, K. L. Mungall, A. M. Cerdeno-Tarraga, L. Temple, K. James, B. Harris, M. A. Quail, M. Achtman, R. Atkin, S. Baker, D. Basham, N. Bason, I. Cherevach, T. Chillingworth, M. Collins, A. Cronin, P. Davis, J. Doggett, T. Feltwell, A. Goble, N. Hamlin, H. Hauser, S. Holroyd, K. Jagels, S. Leather, S. Moule, H. Norberczak, S. O'Neil, D. Ormond, C. Price, E. Rabbinowitsch, S. Rutter, M. Sanders, D. Saunders, K. Seeger, S. Sharp, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, L. Unwin, S. Whitehead, B. G. Barrell, and D. J. Maskell.** 2003.

Comparative analysis of the genome sequences of Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Nat Genet **35:**32-40.

25. **Phalipon, A., C. Costachel, C. Grandjean, A. Thuizat, C. Guerreiro, M. Tanguy, F. Nato, B. Vulliez-Le Normand, F. Belot, K. Wright, V. Marcel-Peyre, P. J. Sansonetti, and L. A. Mulard.** 2006. Characterization of functional oligosaccharide mimics of the Shigella flexneri serotype 2a O-antigen: implications for the development of a chemically defined glycoconjugate vaccine. J Immunol **176:**1686-1694.
26. **Pilione, M. R., and E. T. Harvill.** 2006. The Bordetella bronchiseptica type III secretion system inhibits gamma interferon production that is required for efficient antibody-mediated bacterial clearance. Infect Immun **74:**1043-1049.
27. **Pishko, E. J., G. S. Kirimanjeswara, M. R. Pilione, L. Gopinathan, M. J. Kennett, and E. T. Harvill.** 2004. Antibody-mediated bacterial clearance from the lower respiratory tract of mice requires complement component C3. Eur J Immunol **34:**184-193.
28. **Preston, A., A. G. Allen, J. Cadisch, R. Thomas, K. Stevens, C. M. Churcher, K. L. Badcock, J. Parkhill, B. Barrell, and D. J. Maskell.** 1999. Genetic Basis for Lipopolysaccharide O-Antigen Biosynthesis in Bordetellae. Infect. Immun. **67:**3763-3767.
29. **Preston, A., B. O. Petersen, J. O. Duus, J. Kubler-Kielb, G. Ben-Menachem, J. Li, and E. Vinogradov.** 2006. Complete Structures of Bordetella bronchiseptica and Bordetella parapertussis Lipopolysaccharides. J. Biol. Chem. **281:**18135-18144.
30. **Restif, O., and B. T. Grenfell.** 2006. Integrating life history and cross-immunity into the evolutionary dynamics of pathogens. Proc Biol Sci **273:**409-416.
31. **Restif, O., D. N. Wolfe, E. M. Goebel, O. N. Bjornstad, and E. T. Harvill.** 2008. Of mice and men: asymmetric interactions between Bordetella pathogen species. Parasitology**:**1-13.
32. **Stainer, D. W., and M. J. Scholte.** 1970. A simple chemically defined medium for the production of phase I Bordetella pertussis. J Gen Microbiol **63:**211-220.
33. **Stanislavsky, E. S., T. A. Makarenko, and T. E. Kozhenova.** 1992. Specific and non-specific mouse protection induced by different chemotypes of the Pseudomonas aeruginosa lipopolysaccharides. FEMS Microbiol Immunol **5:**181-189.
34. **van den Akker, W. M.** 1998. Lipopolysaccharide expression within the genus Bordetella: influence of temperature and phase variation. Microbiology **144:**1527-1535.
35. **van der Zee, A., F. Mooi, J. Van Embden, and J. Musser.** 1997. Molecular evolution and host adaptation of Bordetella spp.: phylogenetic analysis using

multilocus enzyme electrophoresis and typing with three insertion sequences. J Bacteriol **179:**6609-6617.

36. **von Koenig, C. H., A. Tacken, and H. Finger.** 1988. Use of supplemented Stainer-Scholte broth for the isolation of Bordetella pertussis from clinical material. J Clin Microbiol **26:**2558-2560.
37. **Watanabe, M., and M. Nagai.** 2004. Whooping cough due to Bordetella parapertussis: an unresolved problem. Expert Rev Anti Infect Ther **2:**447-454.
38. **Wills-Karp, M.** 2007. Complement Activation Pathways: A Bridge between Innate and Adaptive Immune Responses in Asthma. Proc Am Thorac Soc **4:**247-251.
39. **Wolfe, D. N., E. M. Goebel, O. N. Bjornstad, O. Restif, and E. T. Harvill.** 2007. The O Antigen Enables Bordetella parapertussis To Avoid Bordetella pertussis-Induced Immunity. Infect. Immun. **75:**4972-4979.
40. **Wolfe, D. N., G. S. Kirimanjeswara, E. M. Goebel, and E. T. Harvill.** 2007. Comparative Role of Immunoglobulin A in Protective Immunity against the Bordetellae. Infect. Immun. **75:**4416-4422.

Chapter 4: O-antigen Protects *Bordetella parapertussis* from Complement

Abstract

Bordetella pertussis, a causative agent of whooping cough, expresses BrkA which confers serum resistance, but the closely related human pathogen that also causes whooping cough, *B. parapertussis*, does not. Interestingly, *B. parapertussis*, but not *B. pertussis*, produces an O-antigen, a factor shown in other models to confer serum resistance. Using a murine model of infection, we determined that O-antigen contributes to the ability of *B. parapertussis* to colonize the respiratory tract during the first week of infection, but not thereafter. Interestingly, an O-antigen deficient strain of *B. parapertussis* was not defective in colonizing mice lacking the complement cascade. O-antigen prevented both complement component C3 deposition on the surface, and complement mediated killing of *B. parapertussis*. In addition, O-antigen was required for *B. parapertussis* to systemically spread in complement-sufficient mice, but not complement-deficient mice. These data indicate that O-antigen enables *B. parapertussis* to efficiently colonize the lower respiratory tract by protecting against complement-mediated control and clearance.

Introduction

The major component of the outer leaflet of Gram-negative bacteria, LPS, is composed of three major regions; a lipid A, a core oligosaccharide and an O-polysaccharide (O-antigen) (12). Most biological effects of LPS have been attributed to the immunostimulatory properties of lipid A (35, 44), however, O-antigen plays important roles in protecting against host immune mechanisms such as complement-mediated killing, and antimicrobial peptide-mediated bacteriocidal effects (9, 24, 38, 39, 45, 48, 52, 53). For example, the shorten O-antigen of serum-sensitive strains of *Pseudomonas aeruginosa* is associated with increased C3 deposition (47). The presence of O-antigen on *Klebsiella pneumoniae* LPS appears to have no effect on C3 deposition or its ability to cause pneumonia, although it does increase serum resistance *in vitro* (1, 16). In addition, *P. aeruginosa* and *Yersinia entercolitica* O-antigens affect the expression and/or proper function of other virulence factors (4, 6, 10, 38). These examples illustrate that there is considerable variation in the function of the O-antigen portion of the LPS among different bacterial pathogens (12, 35).

Bordetella parapertussis and *B. pertussis* are the causative agents of whooping cough (34). Although these pathogens are very closely related (17, 36, 56), there is substantial variation in LPS structures between them (2, 3, 17, 42, 43). *B. pertussis* produces a lipooligosaccharide (LOS) containing lipid A and a branched-chain core oligosaccharide with a complex trisaccharide modification, but completely lacks O-antigen due to a 20kb deletion in the *wbm* locus responsible for O-antigen synthesis (36). *B. parapertussis* produces an LPS molecule that has a distinct lipid A and a core oligosaccharide lacking the trisaccharide modification, but includes an O-antigen (42, 43, 55). Interestingly, both of these pathogens are endemic in the human population, indicating that O-antigen is not necessary for human infection (34). However, a defined role for O-antigen during *B. parapertussis* infection has not yet been clearly described.

Previous studies have shown that, compared to the wild type strain, an isogenic mutant of *B. parapertussis* lacking several genes necessary for O-antigen synthesis (Δ*wbm*) is severely defective in colonization of the respiratory tracts of BALB/c mice when given in a low dose inoculum (1000 CFU) and is more sensitive to *in vitro* serum-mediated killing (9). This increased sensitivity to *in vitro* serum exposure was abrogated by prior complement depletion, indicating that O-antigen is protective against complement-mediated killing *in vitro* (9).

To investigate the role of O-antigen during infection, we used a standard high dose inoculation regimen (18, 22, 23, 27, 32). This allowed *B. parapertussisΔwbm* to persist in the respiratory tract for 28 days, similar to wild type bacteria. Interestingly, the O-antigen mutant showed a defect only during the first week of infection suggesting that the defect is due to an increased susceptibility to, or an increased activation of, an innate immune function. While *B. parapertussisΔwbm* numbers were lower than wild type *B. parapertussis* in the absence of neutrophils or macrophages, this defect was not

observed in mice deficient in complement component C3. *In vitro* assays showed that O-antigen inhibited complement component C3 deposition on *B. parapertussis*. In $RAG^{-/-}$ mice, O-antigen was required for the systemic spread of *B. parapertussis* but was not required following complement depletion. This suggests that O-antigen facilitates the systemic spread of *B. parapertussis* via evasion of complement. Together, our data indicate that the O-antigen of *B. parapertussis* protects against complement deposition and complement-mediated killing, allowing for efficient colonization of the murine host.

Materials and Methods:

Bacterial Strains and Growth. *B. parapertussis* strain CN2591 and an isogenic mutant lacking O-antigen, CN2591*Δwbm*, have been previously described (42) and were maintained on Bordet-Gengou agar (Difco, Sparks, MD) containing 10% sheep's blood (Hema Resources, Aurora, OR) with 20 μg/ml streptomycin. For inoculation, the bacteria were grown overnight at 37 ºC in Stainer-Scholte broth (26, 54) to mid-log phase and diluted in phosphate buffered saline (PBS, Omnipur, Gibbstown, NJ) to a concentration of 1.0×10^7 cfu/ml (23, 28, 60, 61).

Animal Experiments. 4 to 6 week old C57BL/6, CD11b$^{-/-}$, μMT, RAG1$^{-/-}$ and C5$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were bred in a *Bordetella*-free environment. C3 knockout (C3$^{-/-}$) mice were a kind gift from Dr. Rick Wetsel and have been previously described (15). All mice were maintained in Pennsylvania State University approved housing facilities and were closely monitored in accordance with institutional policies and IACUC regulations. For inoculation, mice were lightly sedated with 5% isofluorane in oxygen and 50 μl of PBS containing 5×10^5 CFU of the indicated bacteria were pipetted onto the tip of the external nares (28, 62). Groups of three or four animals were sacrificed via CO_2 inhalation on the indicated days for post-mortem dissection of the lungs, trachea, nasal cavity, spleen, liver and/or kidneys, as indicated. Bacterial colonization was quantified by homogenizing each tissue in PBS, serial plating and subsequent colony count (22). Serum was collected via post-mortem cardiac puncture from μMT mice. Survival curves were generated by infection of groups of 15 to 20 RAG1$^{-/-}$ mice with either CN2591 or CN2591Δ*wbm* with or without Cobra Venom Factor (CVF) treatment, as indicated (50). Mice suffering from lethal bordetellosis as determined by severe hunched posture, ruffled fur, extremely labored breathing and apathy were euthanized to prevent unnecessary suffering (31, 62).

Depletion of Immune Factors and Quantification of Leukocytes. For neutrophil depletion, mice were intraperitoneally (i.p.) injected with 1 mg αLy-6G antibody, from the hybridoma RB6-8C5, 48 hours prior to inoculation (27, 51). Depletion of 99% of neutrophils was confirmed via CBC/DIFF analysis. For complement depletion, mice were i.p. injected with 5 units of CVF in PBS (Sigma, St. Louis, MO) at 26 and 24 hours prior to inoculation, and every five days thereafter until the completion of the experiment (50). For alveolar macrophage depletion, mice were given an intranasal dose of 100 μl of clodronate-liposomes (Roche Diagnostics, Mannheim) 48 hours prior to inoculation (11). Presence or absence of alveolar macrophage or neutrophils in the lungs was determined via visual identification of cells from BAL fluid spun onto slides via Cytospin and then stained with modified Wright-Giesma stain (Fisher Scientific, Kalamazoo, MI).

C3 Deposition Assay. Approximately 4 x 10^8 CFU of mid-log phase CN2591 and CN2591Δ*wbm* bacteria were harvested by centrifugation and incubated at 37°C for 30 minutes with 20% naïve, antibody-deficient, complement-active or heat-inactivated serum collected from µMT mice. Samples were washed twice with PBS, incubated for 30 minutes on ice with FITC- labeled goat anti-mouse C3 (MP Biomedicals), washed twice with cold PBS to remove unbound antibody and then analyzed by flow cytometry. Samples were run in triplicate and the experiment was independently repeated.

Serum Killing Assay. Naïve C57BL/6 and $C3^{-/-}$ mice were bled and the pooled blood was placed on ice for 30 min. Samples were spun and the plasma was extracted and diluted to the indicated concentrations. 1000 CFU of mid-log phase CN2591 and CN2591Δ*wbm* in 5µL of PBS were incubated with 45µL of diluted plasma for 1 hour at 37°C followed by subsequent plating and colony count. Samples were run in triplicate and the experiment was independently repeated.

***In Vivo* Bioluminescence Imaging of Bacterial Colonization.** The *B. parapertussis* strains C2591 and C2591Δ*wbm* were rendered bioluminescent by the chromosomal insertion of pSS4266 to produce strains BPS1766 (*B. parapertussis::luciferase*) and BPS1768 (*B. parapertussis*Δ*wbm::luciferase*), respectively. Briefly, pSS4266 is a derivative of the chromosomally-integrating promoter assay vector pSS3110 (57) in which the *lacZYA* operon has been replaced by the *luxCDABE* operon of *Photorhabdus luminescens*, derived from pUTminiTn5km*lux* (21). Luciferase expression in pSS4266 is driven by the *fha* promoter of *Bordetella pertussis*. BPS1766 and BPS1768 were used to inoculate littermate $RAG1^{-/-}$ mice intranasally as described above. Mice were subsequently imaged daily in an IVIS-100 apparatus (Xenogen Corp.) according to the manufacturer's instructions.

Statistical Analysis: Student's two-tailed t-test was used to determine statistical significance between experimental groups. Values of $p \leq 0.05$ were considered significant. Error bars represent standard error.

Results

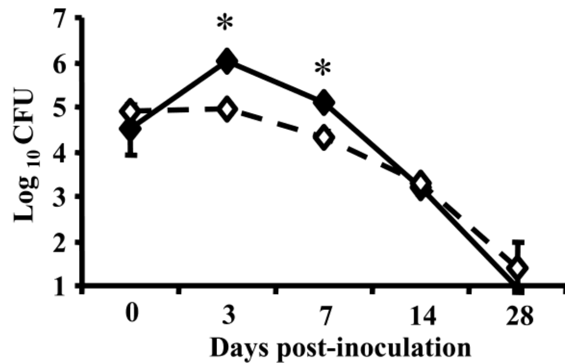

Figure 4.1: **Colonization of mouse lungs by *B. parapertussis* and *B. parapertussisΔwbm* over time.** Groups of three 4-to 6-week-old C57BL/6 mice were inoculated with 5 x 10^5 CFU of *B. parapertussis* (closed diamonds) or *B. parapertussisΔwbm* (open diamonds). The number of CFU recovered from the lungs at each indicated timepoint is expressed as the log_{10} mean ± the standard error. * indicates $P \leq 0.05$. The limit of detection is $Log_{10}1$, indicated as the y-intercept.

O-antigen contributes to *B. parapertussis* colonization of the murine respiratory tract. Previous studies have shown that *B. parapertussis* lacking O-antigen (*B. parapertussisΔwbm*) is nearly cleared from the lower respiratory tract within 3 days using a low dose (1000 CFU) inoculation regimen in BALB/c mice, while *B. parapertussis* is able to increase in numbers (9). To more thoroughly examine interactions between O-antigen and the immune response, we used a high dose inoculation regimen that distributes the bacteria throughout the respiratory tract in numbers that rigorously test the innate and adaptive immune responses (9, 22, 28, 62). C57BL/6 mice were inoculated with 5 x 10^5 CFU of *B. parapertussis* or *B. parapertussisΔwbm,* and sacrificed on days 0, 3, 7, 14, or 28 post-inoculation. Mice dissected 10 minutes post-inoculation had approximately 10^5 CFU of *B. parapertussis* or *B. parapertussisΔwbm* in their lungs (Figure 4.1). *B. parapertussis* numbers increased over the first few days to 10^6 CFU in the lungs and decreased steadily over the subsequent 3 weeks to approximately 10^5 CFU, 10^3 CFU, and 10^1 CFU by days 7, 14 and 28 post-inoculation, respectively (Figure 4.1). *B. parapertussisΔwbm* numbers in the lungs were approximately 10-fold lower than that of *B. parapertussis* on days 3 and 7 post-inoculation, but both *B. parapertussis* and *B. parapertussisΔwbm* were found at similar levels on days 14 and 28 post-inoculation (Figure 4.1). The 90% lower numbers of the O-antigen deficient strain on only days 3 and 7 post-inoculation, but not later days, suggests that the O-antigen of *B. parapertussis* contributes to infection at relatively early stages but has little effect on eventual clearance.

O-antigen does not contribute to colonization in the absence of Complement. In order to investigate the immune mechanism that O-antigen protects against, we blocked various innate immune functions and examined bacterial numbers three days post-inoculation. Neutrophils or alveolar macrophages were depleted from wild type mice with αLy6G antibody or Clodronate-liposome treatment, respectively (11, 27, 51). These mice were then inoculated with *B. parapertussis* or *B. parapertussisΔwbm* as described above, and dissected 3 days post-inoculation. In neutrophil-depleted mice, *B. parapertussis* reached approximately $10^{6.5}$ CFU, while *B. parapertussisΔwbm* reached levels of $10^{5.5}$ CFU in the lungs, a 10-fold defect as compared with wild type bacteria (Figure 4.2). In addition, there was no difference in neutrophil accumulation in the lungs of wild type mice at 12 hours and 24 hours post-inoculation with *B. parapertussis* or *B. parapertussisΔwbm* (data not shown). This indicated that O-antigen contributes to colonization even in the absence of neutrophils, and therefore must protect *B. parapertussis* from some other immune function (Figure 4.2). Similarly, the O-antigen deficient stain was also defective in its ability to colonize alveolar macrophage-depleted mice (Figure 4.2). Unfortunately, mice depleted of both neutrophils and alveolar macrophage succumbed to sham inoculations consisting of sterile PBS, and therefore the effect of O-antigen on the ability of *B. parapertussis* to colonize doubly depleted hosts was not assessed. These results suggest that the early defect of the O-antigen deficient strain is not simply the result of increased susceptibility to neutrophil- or macrophage-mediated clearance alone, but is instead due to sensitivity to some other innate immune function.

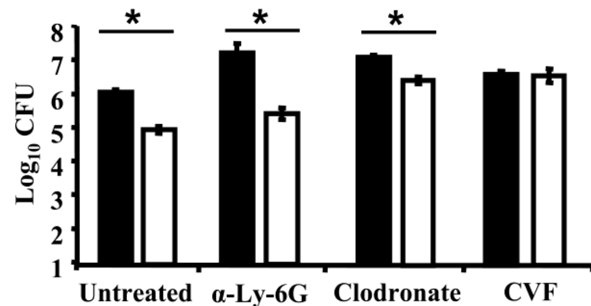

Figure 4.2: **Colonization of mouse lungs by *B. parapertussis* and *B. parapertussisΔwbm* upon depletion of neutrophils, alveolar macrophage or complement.** Groups of three 4-to 6-week-old C57BL/6 mice were left untreated or treated with α-Ly-6G antibodies, Clodronate liposomes, or CVF prior to inoculation with 5 x 10^5 CFU of *B. parapertussis* (closed bars) or *B. parapertussisΔwbm* (open bars), as indicated. The number of CFU recovered from the lungs at day 3 post-inoculation is expressed as the $\log_{10}$ mean ± the standard error. * indicates P ≤ 0.05. The limit of detection is $\text{Log}_{10}1$, indicated as the y-intercept.

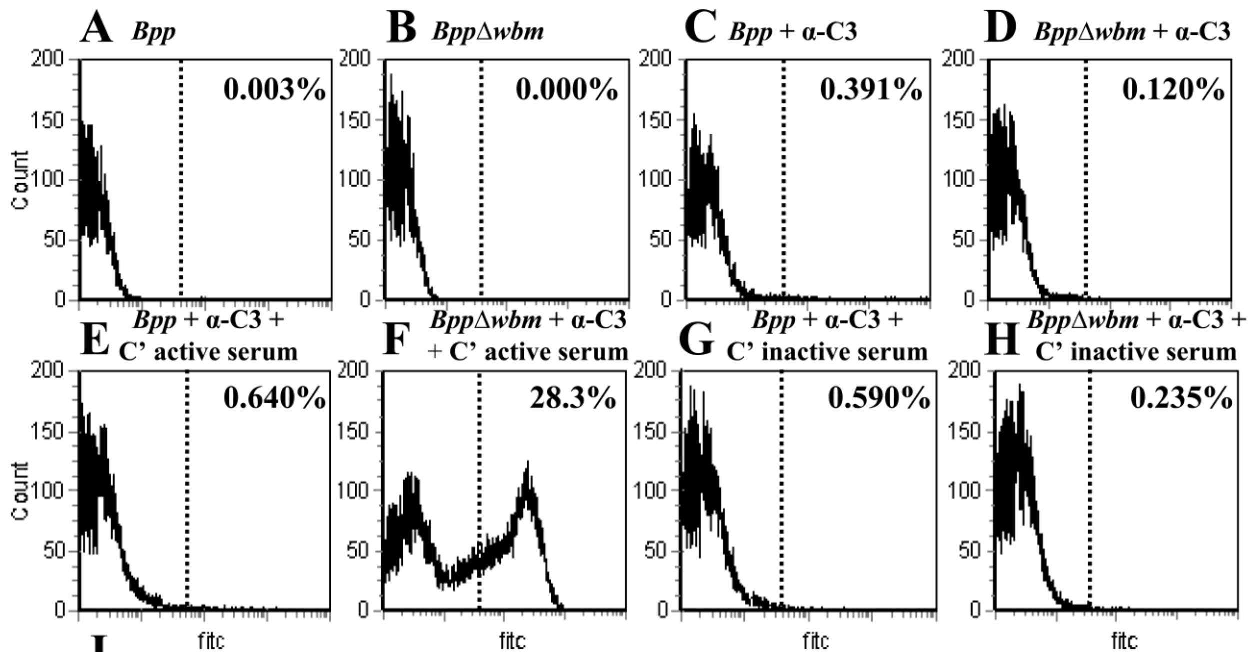

Figure 4.3: Flow cytometry analysis of C3 deposition on *B. parapertussis* and *B. parapertussisΔwbm*. Approximately 4 x 10^8 CFU of *B. parapertussis* (A,C,E,G) or *B. parapertussisΔwbm* (B,D,F,H) were harvested from mid-log phase cultures, incubated at 37°C without serum (A-D), with 20% complement active naïve mouse serum (E-F), or with 20% heat inactivated naive mouse serum (G-H) for 30 minutes. Samples were either left unstained (A-B) or were labeled with FITC-anti-mouse C3 antibodies (C-H), and analyzed by flow cytometry. Percent FITC positive cells are indicated as the average of three replicates. (I) *B. parapertussis* (squares) or *B. parapertussisΔwbm* (triangles) were exposed to complement active (closed) or complement deficient (open) mouse serum at the indicated concentrations for 1 hour. The average percent survival of three replicates is shown. Error bars represent standard error. * indicates $P \leq 0.05$.

Since *B. parapertussisΔwbm* is susceptible to complement *in vitro* (9), we sought to determine if complement was responsible for the defect of *B. parapertussisΔwbm*. Cobra venom factor (CVF) was given to deplete complement in wild type mice 26 and 24 hours prior to inoculation (50). Both *B. parapertussis* and *B. parapertussisΔwbm* were recovered at approximately $10^{6.5}$ CFU on day 3 post-inoculation in the lungs of CVF treated mice, a level similar to that of untreated mice inoculated with wild type *B. parapertussis* (Figure 4.2). These results suggest that the lack of O-antigen leaves the bacteria more susceptible to complement-mediated control *in vivo*. Furthermore, there appears to be no significant role for O-antigen in the absence of complement during the first week of infection (Figure 4.2).

O-antigen prevents C3-deposition on the surface of *B. parapertussis*. Since O-antigen appeared to protect *B. parapertussis* against complement mediated control,

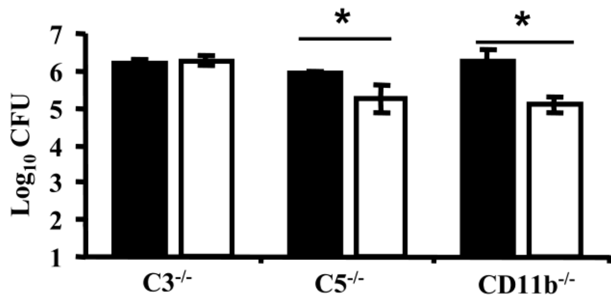

Figure 4.4: C3 mediates *in vivo* control of *B. parapertussisΔwbm*. Groups of three 4-to 6-week-old $C3^{-/-}$, $C5^{-/-}$, or $CD11b^{-/-}$ mice were inoculated with 5 x 10^5 CFU of *B. parapertussis* (closed bars) or *B. parapertussisΔwbm* (open bars). The number of CFU recovered from the lungs at day 3 post-inoculation is expressed as the $\log_{10}$ mean ± the standard error. * indicates $P \leq 0.05$. The limit of detection is $\text{Log}_{10}1$, indicated as the y-intercept.

we sought to examine the ability of C3 to bind to the surface of *B. parapertussis* and *B. parapertussisΔwbm*. Bacteria were incubated with 20% naïve, antibody-deficient serum that was either maintained as complement active or heat-inactivated. Surface bound C3 was detected by FITC labeled anti-C3 antibodies. *B. parapertussis* incubated with serum showed a similar amount of FITC-positive staining as *B. parapertussis* incubated without serum or with heat-inactivated serum (Figure 4.3), indicating that wild type *B. parapertussis* was able to avoid complement deposition. Interestingly, *B. parapertussisΔwbm* showed a 50-fold increase in C3-positive cells (Figure 4.3F) when incubated with complement active serum compared to the number of C3-positive *B. parapertussis* cells with similar treatment (Figure 4.3E). C3 deposition on the O-antigen mutant was not observed upon incubation with heat-inactivated serum (Figure 4.3G-H). Together, these data indicate that the presence of O-antigen prevents C3 deposition on *B. parapertussis*.

To determine if the deposition of C3 caused subsequent complement mediated killing of *B. parapertussisΔwbm*, 1000 CFU of either *B. parapertussis* or *B. parapertussisΔwbm* were incubated with various concentrations of complement active serum from wild type mice or complement deficient serum from $C3^{-/-}$ mice. *B. parapertussisΔwbm* was significantly more sensitive to wild type serum than complement deficient serum at concentrations of 10% serum or higher (Figure 4.3I). These results indicate that O-antigen protects *B. parapertussis* from C3 deposition and subsequent complement mediated killing.

C3 causes the *in vivo* defect of the O-antigen deficient strain of *B. parapertussis*. In order to dissect the specific components of complement that limited *B. parapertussisΔwbm* numbers by day 3 post-inoculation, we inoculated mice lacking different aspects of the complement cascade. $C3^{-/-}$ mice are unable to produce complement component C3, the protein required for positive feedback and amplification of both

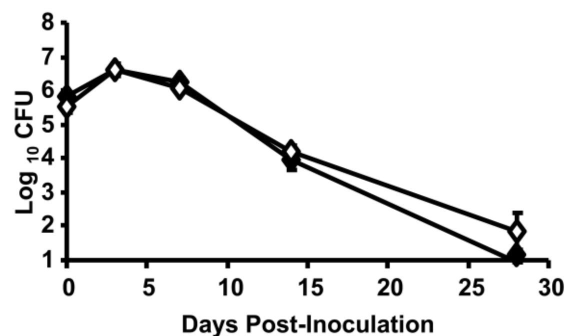

Figure 4.5: *B. parapertussis* colonization in $C3^{-/-}$ mice over time. $C3^{-/-}$ mice were inoculated with either *B. parapertussis* (closed diamonds) or *B. parapertussisΔwbm* (open diamonds). The number of CFU recovered from the lungs on the indicated day is expressed as the $\log_{10}$ mean ± the standard error. * indicates $P \leq 0.05$. The limit of detection is $\text{Log}_{10}1$, indicated as the y-intercept.

classical and alternative complement activation pathways, as well as opsonization and subsequent formation of the membrane attack complex (MAC) on bacterial cells (15). $C3^{-/-}$ mice harbored similar bacterial loads of *B. parapertussis* and *B. parapertussisΔwbm*, indicating that C3 is required to reduce the numbers of *B. parapertussisΔwbm* (Figure 4.4). $C5^{-/-}$ mice lack complement component C5 which is required to form the MAC but not for the positive feedback, amplification and opsonization via C3 deposition. $CDllb^{-/-}$ mice are able to form the MAC complex, but lack the complement receptor type III (CR3) which is known to be important in host response to LPS and is found on macrophages, neutrophils, dendritic cells and natural killer (NK) cells (37, 40, 46, 58). *B. parapertussisΔwbm* numbers were 10-fold lower than wild type bacteria in both $C5^{-/-}$ and $CDllb^{-/-}$ mice, indicating that individually, these complement factors are not required for the more efficient control of *B. parapertussisΔwbm* (Figure 4.4). In addition, a complete timecourse of *B. parapertussis* and *B. parapertussisΔwbm* in $C3^{-/-}$ mice showed that the colonization defect of the O-antigen mutant was abrogated in the absence of complement (Figure 4.5). These results further indicate that the defect in colonization of the O-antigen mutant is a result of its increased susceptibility to complement activities that require C3, but not C5 or CR3 individually.

O-antigen is required for the systemic spread of *B. parapertussis*. Since O-antigen is known to facilitate systemic colonization in other infection models, we sought to determine the role of O-antigen in the systemic spread of *B. parapertussis* (30, 49). $RAG1^{-/-}$ mice were infected with either *B. parapertussis::luciferase* or *B. parapertussis∆wbm::luciferase* and colonization was visualized on day 21 post-inoculation (Figure 4.6A and B). Mice infected with *B. parapertussis::luciferase* showed high levels of colonization in the thoracic cavity, and colonization in the upper abdominal cavity. In contrast, mice infected with *B. parapertussis∆wbm::luciferase* showed colonization only in the respiratory tract. To determine the numbers of *B. parapertussis* or *B. parapertussis∆wbm* in systemic organs, groups of 4-6 $RAG1^{-/-}$ mice were infected with either *B. parapertussis* or *B. parapertussisΔwbm* and then dissected on day 17 post-inoculation. Significantly higher numbers of *B. parapertussis* were found in the respiratory tract compared to *B. parapertussisΔwbm*. Additionally, *B. parapertussis* had spread systemically in all $RAG1^{-/-}$ mice and was found in the spleen, liver and kidneys, whereas no systemic colonization by *B. parapertussisΔwbm* was detected (Figure 4.6C). Groups of 15 – 20 $RAG1^{-/-}$ mice were inoculated with either *B. parapertussis* or *B. parapertussisΔwbm* and monitored for survival. Mice infected with *B. parapertussis* survived for 2 weeks before showing signs of lethal bordetellosis. By day 17 post-inoculation 50% of the mice had died and all mice succumbed to *B. parapertussis* infection by day 21 post-inoculation, consistent with previously reported findings (62). In contrast, all mice inoculated with *B. parapertussisΔwbm* survived until the end of the

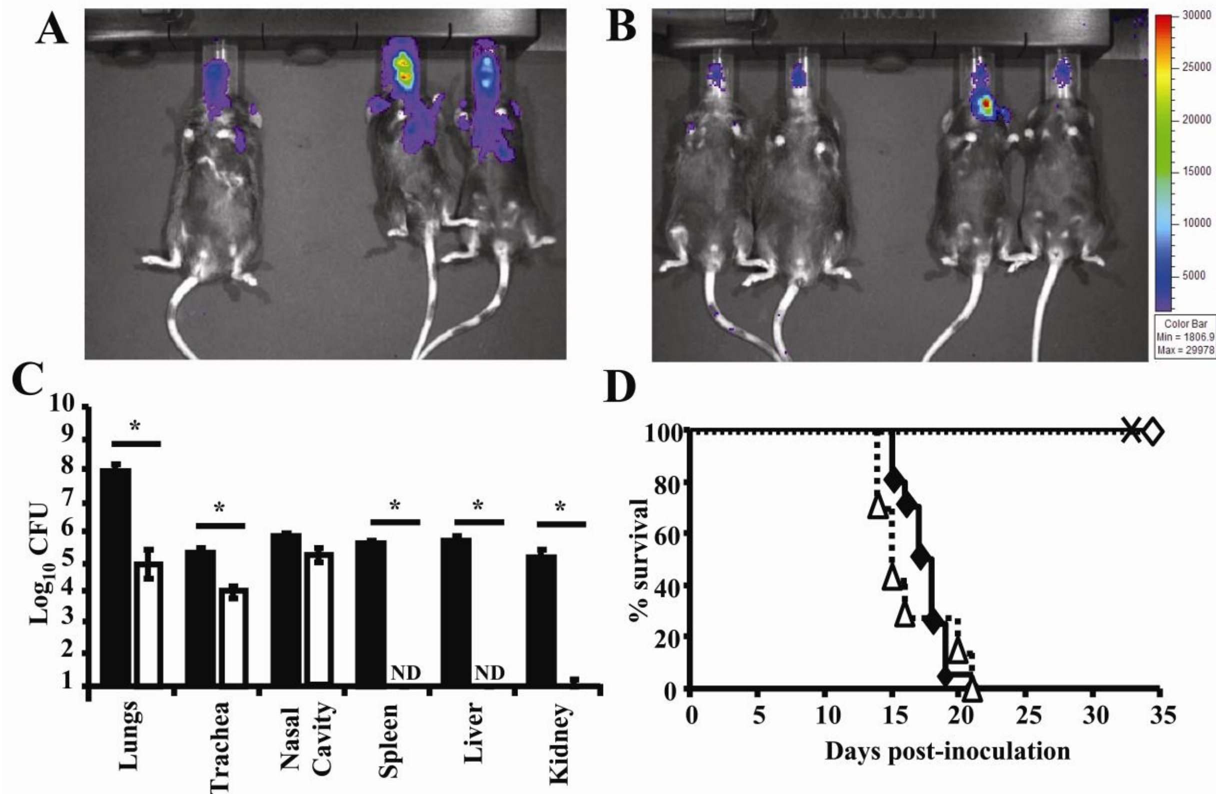

Figure 4.6: Role of O-antigen in the lethality and systemic spread of *B. parapertussis* infections. 4-month old littermate RAG1$^{-/-}$ mice were infected with (A) *B. parapertussis::luciferase* or (B) *B. parapertussisΔwbm::luciferase* and the light production was visualized day 21 post-inoculation and is represented by heat-plot. (C) Groups of three to six 4- to 6-week old RAG1$^{-/-}$ mice were inoculated with *B. parapertussis* (closed bars) or *B. parapertussisΔwbm* (open bars) and were dissected on day 17 post-inoculation for bacterial enumeration in the indicated organs. CFU are expressed as the $\log_{10}$ mean ± the standard error. * indicates P ≤ 0.05. The limit of detection is $\text{Log}_{10}1$, indicated as the y-intercept. ND indicates that no CFU were detected in the sample. (D) Groups of 15 to 20 4- to 6-week old RAG1$^{-/-}$ mice were inoculated with 5 x 10^5 CFU of *B. parapertussis* (closed), *B. parapertussisΔwbm* (open) or sham inoculated (X), with (triangle or X) or without (diamond) CVF treatment, and monitored for survival.

experiment (day 100) without any overt signs of disease (Figure 5.6D and data not shown).

To determine if complement-mediated control of *B. parapertusissisΔwbm* prevented systemic spread and enabled the survival of *B. parapertussisΔwbm* infected mice, groups of RAG1$^{-/-}$ mice were treated with CVF to deplete complement. These mice were then either sham-inoculated or inoculated with *B. parapertussisΔwbm*. CVF treated, sham-inoculated animals showed no sign of deteriorating disease up to day 35. In contrast, CVF treated, *B. parapertussisΔwbm* inoculated animals survived for 2 weeks before showing signs of lethal bordetellosis, a similar survival curve to that of RAG1$^{-/-}$ mice inoculated with *B. parapertussis* (Figure 4.6D). By day 21 post-inoculation, all CVF treated, *B. parapertussisΔwbm* inoculated mice had succumbed to infection (Figure 4.6D). Therefore, the depletion of complement allowed the systemic spread of *B. parapertussisΔwbm*, further indicating that complement mediates the more efficient control of the O-antigen deficient strain.

Discussion

Here we define a specific role for the O-antigen of *B. parapertussis* in a murine model of infection. *B. parapertussisΔwbm* is controlled more efficiently than the wild type bacteria during the first week of infection in wild type mice (Figure 4.1). This defect is dependent on the presence of complement component C3 (Figure 4.2, 4.4 and 4.5) but not neutrophils or macrophages. O-antigen prevents C3 deposition on the surface of *B. parapertussis* (Figure 4.3) and allows for the systemic spread of *B. parapertussis* in immunodeficient mice (Figure 4.6). When complement was depleted, *B. parapertussisΔwbm* was also able to lethally infect immunodeficient mice, indicating that O-antigen is not required for any aspect of infection and systemic spread in the absence of complement. Together these data indicate that an important role of O-antigen in infection and virulence of naïve hosts by *B. parapertussis* is complement resistance.

Activation of the complement cascade results in several different antimicrobial effects including opsonization, neutrophil recruitment scavenger receptor recognition, complement cascade amplification, and formation of the MAC complex (59). In our study we found that *B. parapertussisΔwbm* has no observable defect compared to wild type mice when all of these complement activities were removed ($C3^{-/-}$ or CVF-treated mice), but was defective when individual pathways were eliminated ($C5^{-/-}$, neutrophil-depleted or $CD11b^{-/-}$ mice) (Figure 4.2 and 4.4). This suggests redundancy in the protective mechanisms of complement against *B. parapertussis*.

B. pertussis utilizes the same host population as *B. parapertussis* and causes the same disease, although some studies show that *B. parapertussis*-caused symptoms are milder and/or shorter in duration, other studies suggest that this may not be the case (7, 25, 33). However, *B. pertussis* does not express an O-antigen, which is apparently not crucial to the infection of human hosts by the bordetellae. As the maintenance of O-antigen by *B. parapertussis* confers resistance to complement deposition *in vitro* and complement-mediated control *in vivo*, *B. pertussis* likely developed other mechanisms to protect itself against the complement cascade. For example, BrkA is produced by *B. pertussis* and is known to confer resistance to serum mediated killing (5, 20). In addition, *B. pertussis* is known to bind C4BP and to acquire resistance to complement during growth *in vivo* by some unknown mechanism (41). While O-antigen may not be crucial to *B. pertussis*, evasion of complement-mediated killing by some mechanism may be vital.

In addition to our observations that O-antigen confers resistance to the innate immune function of complement, we have also begun to address the role of *B. parapertussis* O-antigen in the evasion of adaptive immune responses. Our laboratory has recently shown that O-antigen prevents *B. pertussis*-induced antibodies from binding to *B. parapertussis*, allowing *B. parapertussis* to evade *B. pertussis* induced immunity (60). While the inhibition of complement-mediated killing seems to be important to the infection of naïve hosts, the evasion of cross immunity may have

enabled *B. parapertussis* to invade populations in which *B. pertussis* was already endemic (8, 60). Importantly, current vaccines have little effect against *B. parapertussis* (18) and it has been suggested that the prevalence of *B. parapertussis* may have increased since the introduction of acellular pertussis vaccines (29). Mechanisms by which *B. parapertussis* avoids host immunity are of mounting importance due to the recent resurgence of whooping cough over the last two decades, particularly as the contribution of *B. parapertussis* to this resurgence is unclear (13, 14, 19). An understanding of the protective immune response against *B. parapertussis* will be essential to continue to improve whooping cough vaccines.

References:

1. **Alberti, S., G. Marques, S. Camprubi, S. Merino, J. M. Tomas, F. Vivanco, and V. J. Benedi.** 1993. C1q binding and activation of the complement classical pathway by Klebsiella pneumoniae outer membrane proteins. Infect. Immun. **61:**852-860.
2. **Allen, A., and D. Maskell.** 1996. The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in Bordetella pertussis. Mol Microbiol **19:**37-52.
3. **Allen, A. G., R. M. Thomas, J. T. Cadisch, and D. J. Maskell.** 1998. Molecular and functional analysis of the lipopolysaccharide biosynthesis locus wlb from Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Molecular Microbiology **29:**27-38.
4. **Augustin, D. K., Y. Song, M. S. Baek, Y. Sawa, G. Singh, B. Taylor, A. Rubio-Mills, J. L. Flanagan, J. P. Wiener-Kronish, and S. V. Lynch.** 2007. Presence or Absence of Lipopolysaccharide O Antigens Affects Type III Secretion by Pseudomonas aeruginosa. J. Bacteriol. **189:**2203-2209.
5. **Barnes, M. G., and A. A. Weiss.** 2001. BrkA Protein of Bordetella pertussis Inhibits the Classical Pathway of Complement after C1 Deposition. Infect. Immun. **69:**3067-3072.
6. **Bengoechea, J. A., H. Najdenski, and M. Skurnik.** 2004. Lipopolysaccharide O antigen status of Yersinia enterocolitica O:8 is essential for virulence and absence of O antigen affects the expression of other Yersinia virulence factors. Molecular Microbiology **52:**451-469.
7. **Bergfors, E., B. Trollfors, J. Taranger, T. Lagergard, V. Sundh, and G. Zackrisson.** 1999. Parapertussis and pertussis: differences and similarities in incidence, clinical course, and antibody responses. Int J Infect Dis **3:**140-146.
8. **Bjornstad, O. N., and E. T. Harvill.** 2005. Evolution and emergence of Bordetella in humans. Trends Microbiol **13:**355-359.
9. **Burns, V. C., E. J. Pishko, A. Preston, D. J. Maskell, and E. T. Harvill.** 2003. Role of Bordetella O Antigen in Respiratory Tract Infection. Infect. Immun. **71:**86-94.
10. **Campos, M. A., M. A. Vargas, V. Regueiro, C. M. Llompart, S. Alberti, and J. A. Bengoechea.** 2004. Capsule Polysaccharide Mediates Bacterial Resistance to Antimicrobial Peptides. Infect. Immun. **72:**7107-7114.
11. **Carbonetti, N. H., G. V. Artamonova, N. Van Rooijen, and V. I. Ayala.** 2007. Pertussis Toxin Targets Airway Macrophages To Promote Bordetella pertussis Infection of the Respiratory Tract. Infect. Immun. **75:**1713-1720.
12. **Caroff, M., and D. Karibian.** 2003. Structure of bacterial lipopolysaccharides. Carbohydrate Research **338:**2431.
13. **CDC.** 2002. Pertussis--United States, 1997-2000. JAMA **287:**977-979.

14. **Celentano, L. P., M. Massari, D. Paramatti, S. Salmaso, and A. E. Tozzi.** 2005. Resurgence of pertussis in Europe. Pediatr Infect Dis J **24:**761-765.
15. **Circolo, A., G. Garnier, W. Fukuda, X. Wang, T. Hidvegi, A. J. Szalai, D. E. Briles, J. E. Volanakis, R. A. Wetsel, and H. R. Colten.** 1999. Genetic disruption of the murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA. Immunopharmacology **42:**135-149.
16. **Cortes, G., N. Borrell, B. de Astorza, C. Gomez, J. Sauleda, and S. Alberti.** 2002. Molecular Analysis of the Contribution of the Capsular Polysaccharide and the Lipopolysaccharide O Side Chain to the Virulence of Klebsiella pneumoniae in a Murine Model of Pneumonia. Infect. Immun. **70:**2583-2590.
17. **Cummings, C. A., M. M. Brinig, P. W. Lepp, S. van de Pas, and D. A. Relman.** 2004. Bordetella species are distinguished by patterns of substantial gene loss and host adaptation. J Bacteriol **186:**1484-1492.
18. **David, S., R. van Furth, and F. R. Mooi.** 2004. Efficacies of whole cell and acellular pertussis vaccines against Bordetella parapertussis in a mouse model. Vaccine **22:**1892.
19. **de Melker, H. E., J. F. Schellekens, S. E. Neppelenbroek, F. R. Mooi, H. C. Rumke, and M. A. Conyn-van Spaendonck.** 2000. Reemergence of pertussis in the highly vaccinated population of the Netherlands: observations on surveillance data. Emerg Infect Dis **6:**348-357.
20. **Fernandez, R. C., and A. A. Weiss.** 1994. Cloning and sequencing of a Bordetella pertussis serum resistance locus. Infect. Immun. **62:**4727-4738.
21. **Forde, C. B., R. Parton, and J. G. Coote.** 1998. Bioluminescence as a reporter of intracellular survival of Bordetella bronchiseptica in murine phagocytes. Infect Immun **66:**3198-3207.
22. **Harvill, E. T., P. A. Cotter, and J. F. Miller.** 1999. Pregenomic comparative analysis between bordetella bronchiseptica RB50 and Bordetella pertussis tohama I in murine models of respiratory tract infection. Infect Immun **67:**6109-6118.
23. **Harvill, E. T., P. A. Cotter, M. H. Yuk, and J. F. Miller.** 1999. Probing the function of Bordetella bronchiseptica adenylate cyclase toxin by manipulating host immunity. Infect Immun **67:**1493-1500.
24. **Harvill, E. T., A. Preston, P. A. Cotter, A. G. Allen, D. J. Maskell, and J. F. Miller.** 2000. Multiple Roles for Bordetella Lipopolysaccharide Molecules during Respiratory Tract Infection. Infect. Immun. **68:**6720-6728.
25. **Heininger, U., K. Stehr, S. Schmitt-Grohe, C. Lorenz, R. Rost, P. D. Christenson, M. Uberall, and J. D. Cherry.** 1994. Clinical characteristics of illness caused by Bordetella parapertussis compared with illness caused by Bordetella pertussis. Pediatr Infect Dis J **13:**306-309.

26. **Imaizumi, A., Y. Suzuki, S. Ono, H. Sato, and Y. Sato.** 1983. Heptakis(2,6-O-dimethyl)beta-cyclodextrin: a novel growth stimulant for Bordetella pertussis phase I. J Clin Microbiol **17:**781-786.
27. **Kirimanjeswara, G. S., L. M. Agosto, M. J. Kennett, O. N. Bjornstad, and E. T. Harvill.** 2005. Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of Bordetella pertussis. J Clin Invest **115:**3594-3601.
28. **Kirimanjeswara, G. S., P. B. Mann, and E. T. Harvill.** 2003. Role of antibodies in immunity to Bordetella infections. Infect Immun **71:**1719-1724.
29. **Liese, J. G., C. Renner, S. Stojanov, B. H. Belohradsky, and G. The Munich Vaccine Study.** 2003. Clinical and epidemiological picture of B pertussis and B parapertussis infections after introduction of acellular pertussis vaccines. Arch Dis Child **88:**684-687.
30. **Lugo, J. Z., S. Price, J. E. Miller, I. Ben-David, V. J. Merrill, P. Mancuso, J. B. Weinberg, and J. G. Younger.** 2007. Lipopolysaccharide O-antigen promotes persistent murine bacteremia. Shock **27:**186-191.
31. **Mann, P., E. Goebel, J. Barbarich, M. Pilione, M. Kennett, and E. Harvill.** 2007. Use of a Genetically Defined Double Mutant Strain of Bordetella bronchiseptica Lacking Adenylate Cyclase and Type III Secretion as a Live Vaccine. Infect. Immun. **75:**3665-3672.
32. **Mann, P. B., D. Wolfe, E. Latz, D. Golenbock, A. Preston, and E. T. Harvill.** 2005. Comparative toll-like receptor 4-mediated innate host defense to Bordetella infection. Infect Immun **73:**8144-8152.
33. **Mastrantonio, P., P. Stefanelli, M. Giuliano, Y. Herrera Rojas, M. Ciofi degli Atti, A. Anemona, and A. E. Tozzi.** 1998. Bordetella parapertussis infection in children: epidemiology, clinical symptoms, and molecular characteristics of isolates. J Clin Microbiol **36:**999-1002.
34. **Mattoo, S., and J. D. Cherry.** 2005. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin Microbiol Rev **18:**326-382.
35. **Munford, R. S., and A. W. Varley.** 2006. Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria. PLoS Pathog **2:**e67.
36. **Parkhill, J., M. Sebaihia, A. Preston, L. D. Murphy, N. Thomson, D. E. Harris, M. T. Holden, C. M. Churcher, S. D. Bentley, K. L. Mungall, A. M. Cerdeno-Tarraga, L. Temple, K. James, B. Harris, M. A. Quail, M. Achtman, R. Atkin, S. Baker, D. Basham, N. Bason, I. Cherevach, T. Chillingworth, M. Collins, A. Cronin, P. Davis, J. Doggett, T. Feltwell, A. Goble, N. Hamlin, H. Hauser, S. Holroyd, K. Jagels, S. Leather, S. Moule, H. Norberczak, S. O'Neil, D. Ormond, C. Price, E. Rabbinowitsch, S. Rutter, M. Sanders, D. Saunders, K. Seeger, S. Sharp, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, L. Unwin, S. Whitehead, B. G. Barrell, and D. J. Maskell.** 2003.

Comparative analysis of the genome sequences of Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Nat Genet **35:**32-40.

37. **Perera, P.-Y., T. N. Mayadas, O. Takeuchi, S. Akira, M. Zaks-Zilberman, S. M. Goyert, and S. N. Vogel.** 2001. CD11b/CD18 Acts in Concert with CD14 and Toll-Like Receptor (TLR) 4 to Elicit Full Lipopolysaccharide and Taxol-Inducible Gene Expression. J Immunol **166:**574-581.
38. **Perez-Gutierrez, C., C. M. Llompart, M. Skurnik, and J. A. Bengoechea.** 2007. Expression of the Yersinia enterocolitica pYV-Encoded Type III Secretion System Is Modulated by Lipopolysaccharide O-Antigen Status. Infect. Immun. **75:**1512-1516.
39. **Pier, G. B.** 2007. Pseudomonas aeruginosa lipopolysaccharide: A major virulence factor, initiator of inflammation and target for effective immunity. International Journal of Medical Microbiology **297:**277.
40. **Pilione, M. R., L. M. Agosto, M. J. Kennett, and E. T. Harvill.** 2006. CD11b is required for the resolution of inflammation induced by Bordetella bronchiseptica respiratory infection. Cellular Microbiology **8:**758-768.
41. **Pishko, E. J., D. J. Betting, C. S. Hutter, and E. T. Harvill.** 2003. Bordetella pertussis Acquires Resistance to Complement-Mediated Killing In Vivo. Infect. Immun. **71:**4936-4942.
42. **Preston, A., A. G. Allen, J. Cadisch, R. Thomas, K. Stevens, C. M. Churcher, K. L. Badcock, J. Parkhill, B. Barrell, and D. J. Maskell.** 1999. Genetic Basis for Lipopolysaccharide O-Antigen Biosynthesis in Bordetellae. Infect. Immun. **67:**3763-3767.
43. **Preston, A., B. O. Petersen, J. O. Duus, J. Kubler-Kielb, G. Ben-Menachem, J. Li, and E. Vinogradov.** 2006. Complete Structures of Bordetella bronchiseptica and Bordetella parapertussis Lipopolysaccharides. J. Biol. Chem. **281:**18135-18144.
44. **Raetz, C. R. H., C. M. Reynolds, M. S. Trent, and R. E. Bishop.** 2007. Lipid A Modification Systems in Gram-Negative Bacteria. Annual Review of Biochemistry **76:**295-329.
45. **Raynaud, C., K. L. Meibom, M.-A. Lety, I. Dubail, T. Candela, E. Frapy, and A. Charbit.** 2007. Role of the wbt Locus of Francisella tularensis in Lipopolysaccharide O-Antigen Biogenesis and Pathogenicity. Infect. Immun. **75:**536-541.
46. **Ross, G. D.** 2002. Role of the lectin domain of Mac-1/CR3 (CD11b/CD18) in regulating intercellular adhesion. Immunol Res **25:**219-227.
47. **Schiller, N. L., R. A. Hatch, and K. A. Joiner.** 1989. Complement activation and C3 binding by serum-sensitive and serum-resistant strains of Pseudomonas aeruginosa. Infect. Immun. **57:**1707-1713.

48. **Sebastian, S., S. T. Dillon, J. G. Lynch, L. T. Blalock, E. Balon, K. T. Lee, L. E. Comstock, J. W. Conlan, E. J. Rubin, A. O. Tzianabos, and D. L. Kasper.** 2007. A defined O-antigen polysaccharide mutant of Francisella tularensis live vaccine strain has attenuated virulence while retaining its protective capacity. Infect Immun **75:**2591-2602.
49. **Shankar-Sinha, S., G. A. Valencia, B. K. Janes, J. K. Rosenberg, C. Whitfield, R. A. Bender, T. J. Standiford, and J. G. Younger.** 2004. The Klebsiella pneumoniae O Antigen Contributes to Bacteremia and Lethality during Murine Pneumonia. Infect. Immun. **72:**1423-1430.
50. **Shapiro, S., D. O. Beenhouwer, M. Feldmesser, C. Taborda, M. C. Carroll, A. Casadevall, and M. D. Scharff.** 2002. Immunoglobulin G monoclonal antibodies to Cryptococcus neoformans protect mice deficient in complement component C3. Infect Immun **70:**2598-2604.
51. **Sjostedt, A., J. W. Conlan, and R. J. North.** 1994. Neutrophils are critical for host defense against primary infection with the facultative intracellular bacterium Francisella tularensis in mice and participate in defense against reinfection. Infect. Immun. **62:**2779-2783.
52. **Skurnik, M., and J. A. Bengoechea.** 2003. The biosynthesis and biological role of lipopolysaccharide O-antigens of pathogenic Yersiniae. Carbohydrate Research **338:**2521.
53. **Skurnik, M., M. Biedzka-Sarek, P. S. Lubeck, T. Blom, J. A. Bengoechea, C. Perez-Gutierrez, P. Ahrens, and J. Hoorfar.** 2007. Characterization and Biological Role of the O-Polysaccharide Gene Cluster of Yersinia enterocolitica Serotype O:9. J. Bacteriol. **189:**7244-7253.
54. **Stainer, D. W., and M. J. Scholte.** 1970. A simple chemically defined medium for the production of phase I Bordetella pertussis. J Gen Microbiol **63:**211-220.
55. **van den Akker, W. M.** 1998. Lipopolysaccharide expression within the genus Bordetella: influence of temperature and phase variation. Microbiology **144:**1527-1535.
56. **van der Zee, A., F. Mooi, J. Van Embden, and J. Musser.** 1997. Molecular evolution and host adaptation of Bordetella spp.: phylogenetic analysis using multilocus enzyme electrophoresis and typing with three insertion sequences. J Bacteriol **179:**6609-6617.
57. **Veal-Carr, W. L., and S. Stibitz.** 2005. Demonstration of differential virulence gene promoter activation in vivo in Bordetella pertussis using RIVET. Mol Microbiol **55:**788-798.
58. **Wagner, C., G. M. Hansch, S. Stegmaier, B. Denefleh, F. Hug, and M. Schoels.** 2001. The complement receptor 3, CR3 (CD11b/CD18), on T lymphocytes: activation-dependent up-regulation and regulatory function. Eur J Immunol **31:**1173-1180.

59. **Wills-Karp, M.** 2007. Complement Activation Pathways: A Bridge between Innate and Adaptive Immune Responses in Asthma. Proc Am Thorac Soc **4:**247-251.
60. **Wolfe, D. N., E. M. Goebel, O. N. Bjornstad, O. Restif, and E. T. Harvill.** 2007. The O Antigen Enables Bordetella parapertussis To Avoid Bordetella pertussis-Induced Immunity. Infect. Immun. **75:**4972-4979.
61. **Wolfe, D. N., G. S. Kirimanjeswara, E. M. Goebel, and E. T. Harvill.** 2007. Comparative Role of Immunoglobulin A in Protective Immunity against the Bordetellae. Infect. Immun. **75:**4416-4422.
62. **Wolfe, D. N., G. S. Kirimanjeswara, and E. T. Harvill.** 2005. Clearance of Bordetella parapertussis from the Lower Respiratory Tract Requires Humoral and Cellular Immunity. Infect. Immun. **73:**6508-6513.

Chapter 6: Discussion

Implications for the *Bordetella* Field: LPS is a major protective antigen of several gram negative bacteria (18). More specifically, the O-antigen portion of LPS is a protective antigen of some bacteria and is also costly to produce in terms of energy (16). Thus, this molecule must provide some crucial function. In addition to protecting the bacteria from innate host factors such as complement and phagocytosis, the O-antigen also confers serotype specificity (4, 8, 13, 22). Differences in O-antigen serotype have been attributed to the circulation of multiple strains of *Salmonella* (11, 22) in the same host population, suggesting that O-antigen is able to negate the host immune response to shared antigens, via hindering antibody binding (24). Interestingly, *B. pertussis* lost the expression of O-antigen due to a natural 20kb deletion in the *wbm* locus (5), indicating that this factor is not required for success in a human host. However, *B. parapertussis* likely invaded a population in which *B. pertussis* was endemic (2). Thus, this pathogen had to avoid *B. pertussis*-induced immunity to exploit the human host (2). Therefore, creating a distinct serotype from *B. pertussis* via maintaining an O-antigen could have allowed *B. parapertussis* to invade the human population.

Interestingly, the O-antigen of *B. parapertussis* serves two major roles during infection. During a primary infection by *B. parapertussis*, the O-antigen inhibits C3 deposition on the surface of the bacterium; preventing serum mediated killing (Figure 4.3). Without this factor, *B. parapertussis* suffers a 90% decrease in colonization of the lower respiratory tract (Figure 4.5). In addition, the O-antigen facilitates systemic spread of *B. parapertussis* and is required for lethality in immunodeficient mice (Figure 4.6). During a secondary challenge of *B. parapertussis*, a robust antibody response to O-antigen is required for efficient protection (Figures 3.5 and 3.6). While vaccination with a current acellular whooping cough vaccine, the addition of *B. parapertussis* LPS to that vaccine rendered *B. parapertussis* susceptible to the immune response that was induced (Figure 3.7), indicating that some portion of the LPS molecule confers protection against *B. parapertussis*.

The protective immunity conferred by O-antigen could also explain the demographics of *B. parapertussis* infection. *B. parapertussis* is isolated most often from children 5 to 10 years of age, and only very rarely from adults (unpublished data). This pattern could be the result of long-term immunity against protective antigen(s) of *B. parapertussis*, as the majority of cases occur early in life prior to an immunizing exposure. In support of this, subsequent *B. parapertussis* infections have not been observed in a clinical setting. However, no study has examined the duration of *B. parapertussis* immunity in humans, and therefore it is not known if immunity to this pathogen wanes.

B. bronchiseptica also expresses an O-antigen which is antigenically and conformationally distinct from that of *B. parapertussis* (19, 20) due to 7 different genes in the *wbm* locus (5, 17). Similar to *B. parapertussis*, the O-antigen also protects *B. bronchiseptica* from complement mediated killing *in vitro* (3, 19). However, this factor is not required for *in vivo* colonization by *B. bronchiseptica* (3), a difference that could be due to decreased expression of the complement-inhibitory protein BrkA by *B. parapertussis* (23). Interestingly, *B. bronchiseptica* is susceptible to *B. pertussis*-induced immunity (Figures 2.1, 2.6 and 2.7), indicating that the presence of O-antigen does not confer protection against this pathogen. While *B. bronchiseptica* is commonly

found in a wide range of mammals (15), it appears to be associated with immunodeficient humans (25) and is rarely isolated from immunocompetent humans. This phenomenon could be attributed opportunistic infections in humans that lack an effective immune response to *B. pertussis* and therefore are susceptible to zoonotic infections by *B. bronchiseptica*.

Implications for the Immunology Field: Cross-reactive antigens are common. Indeed, cross-reactive antigens are found not only in closely related sources such as cow pox and small pox, but also extremely divergent sources, such as *Staphylococcus aureus* and human heart valve antigens (22). This cross-reactivity can exclude a pathogen like small pox from one population, such as cow-pox exposed milk-maids, while allowing the pathogen to flourish in susceptible populations, such as non-agricultural city-dwellers. However, pathogens are constantly evolving and have developed methods to avoid cross-reactivity. For example, evolution of the hemagglutinin and neuraminidase genes allows multiple influenza strains to co-circulate in one population (7). While cross-reactive antigens may confer a protective immune response against a range of pathogens, this is not always the case for the closely related bacterial species, *B. bronchiseptica, B. pertussis* and *B. parapertussis*.

B. bronchiseptica, a pathogen normally found in non-human mammals, is susceptible to *B. pertussis* infection and vaccination induced immunity. This is mediated by at least two strongly recognized antigens, pertactin and filamentous hemagglutinin (Figures 2.5 and 2.6). Because current human clinical isolates of *B. bronchiseptica* are also sensitive to *B. pertussis*-induced immunity (Figure 2.7), this suggests that *B. bronchiseptica* has not become antigenically distinct to the point where *B. pertussis*-induced immunity is not longer effective.

B. parapertussis, however, is resistant to *B. pertussis*-induced immunity (Figure 3.1) (6). While *B. parapertussis* and *B. pertussis* do share cross-reactive antigens (24), *B. parapertussis* expresses an O-antigen which hinders the binding of *B. pertussis*-induced antibodies to the bacterial surface (24). Only in the presence of O-antigen-specific antibodies is *B. parapertussis* colonization significantly reduced (Figure 4.5). Therefore, it is important to induce immunity to the protective, not just prominent and cross-reactive, antigens.

Implications for the Human Health Field: Zoonoses are a constant source for the evolution and emergence of potential human pathogens (22). Several pathogens, such as the H5N1 stains of Avian Influenza, are constantly monitored and can cause fatal disease in humans (WHO Flunet). However, in the case of H5N1, transmission between humans has not been observed and infections are sporadic zoonotic events. The danger resides in the rapid evolution of this virus, potentially creating a strain that is able to transmit from human to human and cause an epidemic.

Current human pathogens or normal flora may protect humans from invading zoonoses by inducing cross-reactive immunity, such as *B. pertussis* and *B. bronchiseptica*, or by competing for a niche environment such as enteric *E. coli* subspecies (10, 21, 22). *B. bronchiseptica* is susceptible to *B. pertussis*-induced immunity, and therefore may not transmit between humans due to the endemicity of *B. pertussis* and extensive use of whooping cough vaccines. Indeed, the majority of

reported *B. bronchiseptica* cases have been observed in immunocompromised humans (25), who would also lack an effective immune response to *B. pertussis*. This unintended, yet beneficial, effect supports the continued vaccination against *B. pertussis* despite the monetary cost and waning immunity.

While current whooping cough vaccines protect against both *B. bronchiseptica* and *B. pertussis*, they are woefully ineffective against *B. parapertussis* (6). *B. parapertussis* disease may be milder on average than that caused by *B. pertussis*, but it is clinically indistinguishable from *B. pertussis* (1, 9, 12, 14). Indeed, the ability to avoid whooping cough vaccine immunity has allowed *B. parapertussis* to flourish, causing a proportionately higher case load in vaccinated verses unvaccinated individuals (12). Therefore, the addition of a protective antigen of *B. parapertussis*, such as O-antigen, to current whooping cough vaccines may help decrease disease burden. Unfortunately, the addition of whole LPS to a current vaccine is highly unlikely. LPS is a TLR4 agonist and can cause unnecessary inflammation and, in extreme cases, endotoxic shock (16). In addition, public opinion of LPS is very negative, partially due to the alias, endotoxin. However, the immunostimulatory portion of LPS, Lipid A, can be cleaved from the O-antigen, and the purified O-antigen may be added to the vaccine in a conjugated form, thereby reducing the risk of detrimental reactigenicity.

Conclusion: The *Bordetella* are important respiratory pathogens which cause significant morbidity and mortality worldwide. While current human whooping cough vaccines are protective against *B. bronchiseptica* and *B. pertussis*, *B. parapertussis* is able to evade this immunity. The addition of *B. parapertussis* protective antigen(s), such as O-antigen, to current vaccines could result in decreased disease burden, particularly in young children. Further investigations into the bordetellae may reveal other protective antigens that could also be incorporated into current whooping cough vaccines, further increasing vaccine efficacy and reducing the incidence of this disease.

References:

1. **Bergfors, E., B. Trollfors, J. Taranger, T. Lagergard, V. Sundh, and G. Zackrisson.** 1999. Parapertussis and pertussis: differences and similarities in incidence, clinical course, and antibody responses. Int J Infect Dis **3:**140-146.
2. **Bjornstad, O. N., and E. T. Harvill.** 2005. Evolution and emergence of Bordetella in humans. Trends Microbiol **13:**355-359.
3. **Burns, V. C., E. J. Pishko, A. Preston, D. J. Maskell, and E. T. Harvill.** 2003. Role of Bordetella O Antigen in Respiratory Tract Infection. Infect. Immun. **71:**86-94.
4. **Campos, M. A., M. A. Vargas, V. Regueiro, C. M. Llompart, S. Alberti, and J. A. Bengoechea.** 2004. Capsule Polysaccharide Mediates Bacterial Resistance to Antimicrobial Peptides. Infect. Immun. **72:**7107-7114.
5. **Cummings, C. A., M. M. Brinig, P. W. Lepp, S. van de Pas, and D. A. Relman.** 2004. Bordetella species are distinguished by patterns of substantial gene loss and host adaptation. J Bacteriol **186:**1484-1492.
6. **David, S., R. van Furth, and F. R. Mooi.** 2004. Efficacies of whole cell and acellular pertussis vaccines against Bordetella parapertussis in a mouse model. Vaccine **22:**1892.
7. **Finkelman, B. S., C. Viboud, K. Koelle, M. J. Ferrari, N. Bharti, and B. T. Grenfell.** 2007. Global patterns in seasonal activity of influenza A/H3N2, A/H1N1, and B from 1997 to 2005: viral coexistence and latitudinal gradients. PLoS ONE **2:**e1296.
8. **Goebel, E. M., D. N. Wolfe, K. Elder, S. Stibitz, and E. T. Harvill.** 2008. O antigen protects Bordetella parapertussis from complement. Infect Immun **76:**1774-1780.
9. **Heininger, U., K. Stehr, S. Schmitt-Grohe, C. Lorenz, R. Rost, P. D. Christenson, M. Uberall, and J. D. Cherry.** 1994. Clinical characteristics of illness caused by Bordetella parapertussis compared with illness caused by Bordetella pertussis. Pediatr Infect Dis J **13:**306-309.
10. **Huang, D. B., A. Mohanty, H. L. DuPont, P. C. Okhuysen, and T. Chiang.** 2006. A review of an emerging enteric pathogen: enteroaggregative Escherichia coli. J Med Microbiol **55:**1303-1311.
11. **Khakhria, R., D. Woodward, W. M. Johnson, and C. Poppe.** 1997. Salmonella isolated from humans, animals and other sources in Canada, 1983-92. Epidemiol Infect **119:**15-23.
12. **Liese, J. G., C. Renner, S. Stojanov, B. H. Belohradsky, and G. The Munich Vaccine Study.** 2003. Clinical and epidemiological picture of B pertussis and B parapertussis infections after introduction of acellular pertussis vaccines. Arch Dis Child **88:**684-687.
13. **Lugo, J. Z., S. Price, J. E. Miller, I. Ben-David, V. J. Merrill, P. Mancuso, J. B. Weinberg, and J. G. Younger.** 2007. Lipopolysaccharide O-antigen promotes persistent murine bacteremia. Shock **27:**186-191.
14. **Mastrantonio, P., P. Stefanelli, M. Giuliano, Y. Herrera Rojas, M. Ciofi degli Atti, A. Anemona, and A. E. Tozzi.** 1998. Bordetella parapertussis infection in children: epidemiology, clinical symptoms, and molecular characteristics of isolates. J Clin Microbiol **36:**999-1002.

15. **Mattoo, S., and J. D. Cherry.** 2005. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin Microbiol Rev **18:**326-382.
16. **Munford, R. S., and A. W. Varley.** 2006. Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria. PLoS Pathog **2:**e67.
17. **Parkhill, J., M. Sebaihia, A. Preston, L. D. Murphy, N. Thomson, D. E. Harris, M. T. Holden, C. M. Churcher, S. D. Bentley, K. L. Mungall, A. M. Cerdeno-Tarraga, L. Temple, K. James, B. Harris, M. A. Quail, M. Achtman, R. Atkin, S. Baker, D. Basham, N. Bason, I. Cherevach, T. Chillingworth, M. Collins, A. Cronin, P. Davis, J. Doggett, T. Feltwell, A. Goble, N. Hamlin, H. Hauser, S. Holroyd, K. Jagels, S. Leather, S. Moule, H. Norberczak, S. O'Neil, D. Ormond, C. Price, E. Rabbinowitsch, S. Rutter, M. Sanders, D. Saunders, K. Seeger, S. Sharp, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, L. Unwin, S. Whitehead, B. G. Barrell, and D. J. Maskell.** 2003. Comparative analysis of the genome sequences of Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Nat Genet **35:**32-40.
18. **Pier, G. B.** 2007. Pseudomonas aeruginosa lipopolysaccharide: A major virulence factor, initiator of inflammation and target for effective immunity. International Journal of Medical Microbiology **297:**277.
19. **Preston, A., A. G. Allen, J. Cadisch, R. Thomas, K. Stevens, C. M. Churcher, K. L. Badcock, J. Parkhill, B. Barrell, and D. J. Maskell.** 1999. Genetic Basis for Lipopolysaccharide O-Antigen Biosynthesis in Bordetellae. Infect. Immun. **67:**3763-3767.
20. **Preston, A., B. O. Petersen, J. O. Duus, J. Kubler-Kielb, G. Ben-Menachem, J. Li, and E. Vinogradov.** 2006. Complete Structures of Bordetella bronchiseptica and Bordetella parapertussis Lipopolysaccharides. J. Biol. Chem. **281:**18135-18144.
21. **Qadri, F., A. M. Svennerholm, A. S. Faruque, and R. B. Sack.** 2005. Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev **18:**465-483.
22. **Roth, J. A., Brogden, K.A., Stanton, T.B.** 2000. Virulence Mechanisms of Bacterial Pathogens, 3rd ed. ASM Press.
23. **Stefanelli, P., M. Sanguinetti, C. Fazio, B. Posteraro, G. Fadda, and P. Mastrantonio.** 2006. Differential in vitro expression of the brkA gene in Bordetella pertussis and Bordetella parapertussis clinical isolates. J Clin Microbiol **44:**3397-3400.
24. **Wolfe, D. N., E. M. Goebel, O. N. Bjornstad, O. Restif, and E. T. Harvill.** 2007. The O Antigen Enables Bordetella parapertussis To Avoid Bordetella pertussis-Induced Immunity. Infect. Immun. **75:**4972-4979.
25. **Woolfrey, B. F., and J. A. Moody.** 1991. Human infections associated with Bordetella bronchiseptica. Clin Microbiol Rev **4:**243-255.

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Wolfe, D. N., G. S. Kirimanjeswara, **E. M. Goebel,** and E. T. Harvill**.** 2007. "Comparative Role of Immunoglobulin A in Protective Immunity against the Bordetellae." *Infect. Immun*. 75**:**4416-4422.

Wolfe, D. N., **E. M. Goebel,** O. N. Bjornstad, O. Restif, and E. T. Harvill**.** 2007. "The O Antigen Enables Bordetella parapertussis To Avoid Bordetella pertussis-Induced Immunity." *Infect. Immun*. 75**:**4972-4979.

Restif, O., D. N. Wolfe, **E. M. Goebel**, O. N. Bjornstad, and E. T. Harvill. 2008. "Of mice and men: asymmetic interactions between the *Bordetella* pathogen species." *Parasitology.*

Goebel, E. M., D. N. Wolfe, K. Elder, S. Stibitz, and E. T.Harvill. 2008. "O-Antigen Protects *B. parapertussis* from Complement." *Infect. Immun*. 76:1774-1780.