IMPORTANT NOTE: PLEASE READ STATEMENT REGARDING REPLICATION EFFORTS OF FINDINGS IN THIS DISSERTATION INSERTED BETWEEN PAGES 39 AND 40.

Characterization of the Capsular Polysaccharide of *Haemophilus parasuis* and its Application in the Diagnosis and Prevention of Glässer's Disease

Anne Catherine Michalenka Hyman

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

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Thomas J. Inzana, Chair
Clayton Caswell
Tanya LeRoith
Sharon Witonsky

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ABSTRACT

Haemophilus parasuis is a Gram-negative bacterium responsible for Glässer's Disease in pigs, though little is known regarding its antigenic or virulence factors. Our goals were to characterize the *H. parasuis* capsular polysaccharide (CP), determine its role in serotype-specificity and virulence, determine if CP is immunogenic, and develop diagnostic and protective products to prevent rampant *H. parasuis* infection within swine herds. Material from *H. parasuis* was purified using carbohydrate isolation techniques and compared to CPs from other Pasteurellaceae. Rabbits were immunized with CPs to generate antisera for microscopy, immunoassays, and bactericidal assays. CP antisera were conjugated to latex particles to create an agglutination assay for detection and typing of *H. parasuis*. CP was conjugated to Cholera Toxin B, and used to immunize mice and piglets before challenge with *H. parasuis* to determine its protective efficacy against Glässer's Disease. Broth-grown cells expressed CP, which reacted with antisera in microscopy and immunoassays. Broth-grown *H. parasuis* cells were serum-resistant unless homologous anti-CP serum was present. In contrast, agar-grown cells did not react with antisera in immunoassays, and cells were susceptible to killing by normal swine serum. CP was not expressed on the surface of agar-grown cells unless supplemented with bicarbonate. The addition of bicarbonate also contributed to the variability in CP quantity and upregulation of genes in the CP locus. Sensitized latex particles agglutinated strongest with homologous *H. parasuis* CPs, cells, and agar-grown cell lysates, but also reacted weakly with higher concentrations of heterologous CPs. The latex beads did not agglutinate with non-*H. parasuis* swine bacterial pathogens. Mice immunized with the CP-CTB conjugate produced a significantly higher IgG_2/Th_2 response than unimmunized mice or mice immunized with only CP, and immunized mice had fewer bacteria in their tissues that unimmunized mice. The CP conjugate produced a robust IgG antibody response to CP when used to immunized piglets, but because the control animals also survived *H. parasuis* challenge, the protective efficacy remains inconclusive. Therefore, the *H. parasuis* CP is the antigen that confers serotype identity, and can be implemented in methods and help direct future research in disease prevention and serotype tracking in *H. parasuis* infections.

Dedication

To my Mother, Judith Estelle Michalenka, a Human Paradox:

As with many mother-daughter relationships, a complete understanding of the elder by the younger takes many, many years to formulate. More often than not, especially in adolescent years, a daughter can think of her mother as contrarian, an antithesis to human development and progress. But with you, Mom, my understanding of all that you were includes appreciating how you lived your life as a paradox:

As the educator, who always put learning first and believed that knowledge unlocked innumerate doors, your greatest lessons were taught outside of the classroom.

As the accountant, who should have measured success in numbers- figures analyzed, money saved, and salary earned- you instead modestly celebrated by reflecting on the number of people that you helped over the course of your career.

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Attributions

Several colleagues contributed to this dissertation project, including researching, writing, and editing of all the chapters of this dissertation:

Chapters 2-4

Thomas Inzana, Ph.D. (Tyler J. and Frances F. Young Chair of Bacteriology, Department of Biomedical Sciences and Pathobiology at the Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech Carilion Research Institute) is the corresponding author on these manuscripts. He assisted in the project development, writing, and editing of these manuscripts.

Chapter 2

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List of Abbreviations

-FBS: Media without FBS supplement
-O2: Media grown in the absence of oxygen
µg: Microgram
µL: Microliter
Abs: Absorption
ADH: Adipic Acid Dihydrazide
App: *Actinobacillus pleuropneumoniae*
ApJ45: App serotype 1, strain J45
ApJ45-C: J45 without CP
BCA: Bicinchononic Acid Assay
BHI: Brain Heart Infusion
br: Broth medium
br24h: broth culture grown for 24 h
C: Celsius
cDNA: Complimentary DNA
CFU: Colony Forming Units
cm: Centimeter
CP: Capsular Polysacchride
cs: Material collected from cell surface in CP isolation
Ct: Cycle threshold
depC: Diethylpyrocarbonate
DNA: Deoxyribonucleic Acid
EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
ELISA: Enzyme-Linked Immunosorbent Assay
FBS: Fetal Bovine Serum
FITC: Fluorescein isothiocyanate
g: Gram
g: Gravity
GS-MS: Gas Chromatography Mass Spectrometry
h: Hour
HPLC: High Pressure Liquid Chromatography
IF: Immunofluorescent Microscopy
IgG: Immunoglobin G
IgM: Immunoglobin M
Hps: *Haemophilus parasuis*
Hps #: Specific serotype of *Haemophilus parasuis*
HRP: Horseradish Peroxidase
Hs: *Histophilus somni*
kV: Kilovolt
LAA: Latex Agglutination Assay
LOS: Lipooligosaccharide
LPS: Lipopolysaccharide
mg: Milligram

min: Minute
mL: Millileter
M: Molar
mM: Millimolar
MS: Mass Spectrometry
NAD: Nicotinamide Adenine Dinucleotide
NADC: National Animal Disease Center
NaOAc: Sodium Acetate
NFDM: Nonfat Dry Milk
p: material scraped from whole cells grown on solid agar medium
p11b: Hps cells passaged in broth medium eleven times
p11p: Hps cells passaged on solid agar medium eleven times
PBS: Phosphate Buffered Saline
PBS-M: PBS containing magnesium chloride
PBST: Phosphate Buffered Saline with Tween-20
PCM: PBS supplemented with magnesium chloride and calcium chloride
PCR: Polymerase Chain Reaction
PCS: Precolostral Calf Serum
PPLO: Pleuropneumoniae-Like Organism
$PPLO^{+}$: PPLO media supplemented with 5% FBS, 1% glucose, and 100µg/mL NAD
PRRSV: Porcine Reproductive and Respiratory Syndrome Virus
PS: Naïve swine serum
qRT-PCR: Quantitative Real Time Polymerase Chain Reaction
RNA: Ribonucleic Acid
RNASeq: RNA Sequencing
s: material collected from Supt
supt: Supernatant
TEM: Transmission Electron Microscopy
TLC: Thin Layer Chromatography
TMB: 3,3',5,5'-Tetramethylbenzidine
vs: Versus
wc: Whole cells
x: Times

Chapter 1

History and Impact of *Haemophilus parasuis* and the Challenges of Research Regarding Glässer's Disease

Anne C. Michalenka Hyman[1]

[1]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia, USA.

1.1 Introduction

Haemophilus parasuis is a Gram-negative, pleomorphic bacterium belonging to the Pasteurellaceae family of bacteria [1]. While it is typically found as a commensal organism inhabiting only swine, it is responsible for rapid and rampant decimation of swine herds globally, causing Glässer's Disease (polyserositis) in piglets and pneumonia in adult pigs from clean herds [2]. Factors contributing to pathogenesis are commonly stressors such as weaning, transport, or recovery from prior viral or mycoplasma infection [1]. However, the mechanisms responsible for pathogenesis are still largely unknown. Once the bacteria disseminate from the oropharynx to become systemic, the infection becomes highly pathogenic, contagious, and often fatal. Production farms with outbreaks of *H. parasuis* infection can lose up to 60% of its herd within three days, proving costly to the producers [3]. Understanding the cause of dissemination of *H. parasuis* could allow production farmers to stave off infections by preventing such external stimuli.

The current prophylactic treatment in North America is the inclusion of β-lactam antibiotics in the feed supply [4]. However, public health concerns are mounting over the abuse of antibiotics, potentially creating resistant "superbugs" that would be impervious to any treatments [5]. β-lactams and fluoroquinolones are the current treatments for *H. parasuis* [2]. However, proper diagnosis of infection requires samples from compromised animals sent to a clinical laboratory to be grown on agar medium supplemented with nicotinamide adenine dinucleotide and in the presence of carbon dioxide [6]. If further characterization is needed, PCR can be used to confirm the presence of *H. parasuis* [7]. In this timeframe, most of a herd could be compromised with Glässer's Disease. A more rapid and sensitive diagnostic test used in the

field by veterinarians is needed, and could prevent spread of contagious *H. parasuis* within a herd, saving the producers money and resources.

A specific and robust immune response would be the best prevention against *H. parasuis* infection, and commercially available vaccines are available for this cause [8]. However, out of the fifteen serotypes that have been determined, the available vaccines are only designed against three of the known serotypes, and do not confer protection against all virulent serotypes. Moreover, the vaccines are constructed using either live attenuated bacteria or bacterins [9], which contain endotoxin that causes harmful side effects to piglets. These vaccines exist in their current form because the protective serotype-specific antigen for *H. parasuis* has yet to be defined. An antigen-specific subunit vaccine, like those developed for other Pasteurellaceae bacteria [10, 11], would provide a specific and safe method of protection against *H. parasuis* infections in swine herds.

My dissertation research examines the capsular polysaccharide of *H. parasuis*, and how its expression and production are regulated by external stimuli. This is the first report confirming the serotype-specific antigen for *H. parasuis,* and further research regarding the regulation of capsular polysaccharide expression could provide valuable insights into the virulence and transmission contagion of this bacterium. With the knowledge of capsular polysaccharide as the serotype-specific antigen, my research also evaluates its potential use in a developing a diagnostic and serotyping assay, and as a vaccine to prevent Glässer's Disease in piglets.

1.2 *Haemophilus parasuis*

1.2.1 History and description of *H. parasuis*

Haemophilus parasuis is a bacterium that was first observed in 1913 by K. Glässer, who proposed that a small Gram-negative "bacterial rod" was the causative agent for arthritis in young piglets [12]. The bacterium was first isolated in 1931 by Lewis and Shope from a pig with influenza [13], and was named *Haemophilus influenzae suis*, as it was only known at that time to directly associate with the "swine influenza etiological complex" [13]. After initial studies were completed on *Haemophilus influenza suis*, it was documented in 1943 that the bacterium was capable of causing severe disease in swine without the presence of the influenza virus, but was able to enhance the effects of influenza infection and cause severe disorders to the pig [14]. The bacterium named was shortened to *Haemophilus suis* to reflect these findings. Through the development of more thorough and complex taxonomic designations for the genus *Haemophilus*, the current name *Haemophilus parasuis* was conferred in 1976. [15]

The original findings concerning *H. parasuis* may vary from current knowledge, because the bacterium takes on many different characteristics in morphology, virulence, and behavior. *H. parasuis* is a pleomorphic bacterium in the Pasteurellaceae family of the protobacteria phylum. Its cell morphologies vary from short, small coccobacilli to long, filamentous bodies. Its genome size varies between 1.3-2.3 million base pairs containing between 1300 and 2300 open reading frames [16], depending on the specific strain. These genes consist of loci for replication, transcription, bacterial structure, lipooligosaccharide synthesis and transport [17], capsular polysaccharide and exopolysaccharide synthesis and transport [18, 19], and other genes that may be responsible for virulence [20]. Although the machinery for toxins is contained in the genome [21], the role of exotoxins in *H. parasuis* virulence has not been well characterized [22].

1.2.2 History and Advances of Modern Swine Production

Throughout the course of this early exploration of *H. parasuis*, the swine industry began expanding, and local production farms yielded to larger, centralized production facilities. Changes in infrastructure included refrigerated railroad and tractor trailer transportation, and designated indoor production facilities with higher health standards. These improvements allowed for higher production rates in smaller, more cost-effective scenarios, and the opportunity to breed larger, disease-resilient, and higher-meat quality animals [23]. This expansion and refinement culminated in the 1980s, with the official standardization of the swine industry in the United States, creating the modern and current swine production guidelines for swine-specific facilities. As of 2001, over 75% of swine production farms in the United States maintained over 2,000 hogs [24].

While centralization of the swine production and distribution facilities increased profitability for the pork industry, the impact of concentrating pathogens and diseases in these facilities had a devastating effect on production animals. The emergence of a new pathogen, such as the recent outbreak of porcine epidemic diarrhea virus, or PEDV, can decimate the animals in a large facility. The 2013 outbreak of PEDV in the United States infected and killed an estimated 10,000 pigs that tested positive for the virus across 30 states [25] since its onset. The formation of Swine Health Committees within government agencies has allowed producers, scientists and agricultural health officials to pool resources and research findings to establish methods to stave off porcine infections. Such protocols include introducing antibiotics into feed, routine health checks and culling of compromised animals [26]. The health and well-being of production animals is paramount, but the impact on the quality of the meat product is still of large concern to production facilities.

1.2.3 Global agricultural and economic impact of *H. parasuis*

H. parasuis is a pathogen that is not geographically restricted; its prevalence can be traced to Europe, especially Spain and Germany, as well as Brazil and China, as pork is the most consumed meat product in the world. Swine were originally domesticated and bred for meat in ancient Turkey and China, and were crudely transported all over the world when methods of transportation developed, and humanity shifted from a hunting society to an agricultural one [27]. In modern times, technological advances improved methods of transporting animals, and as a result pathogens that effect these production animals have been dispersed all over the world. *H. parasuis* resides in 30-40% of swine in North America in either subclinical or infectious capacity, and the most prevalent pathogenic strains are from serotypes 4, 5, 13, and 14 [28]. Other countries such as China and Spain also exhibit this frequency of occurrence of *H. parasuis* in their swine, and the bacterium also occurs in wild boars outside of North America [6, 29]. However, the amount of swine produced in these countries far exceeds that of North America [30, 31], and *H. parasuis* infection is even more economically devastating to these producers. At the time of this publication, the price of hog meat is $0.6184/pound [32] and the average weight per hog carcass is 209 lbs. If the average American swine producer has 2000 hogs in a facility, and an outbreak of *H. parasuis* infection occurs in the herd, the producer could lose up over $230,000 if the infection is not controlled within three days [33]. The economic devastation increases for producers in other countries where swine production is more prevalent. The need for understanding, controlling, and preventing *H. parasuis* infections globally is necessary for preserving swine health and global agricultural economies.

1.3 Glässer's Disease

1.3.1 Pathogenesis and Hallmarks of Glässer's Disease

H. parasuis is a commensal organism that resides in the oropharynx of swine without causing disease [2]. The bacteria are introduced to piglets after birth via snout-to-snout contact with their sow [1] and remain there until an event triggers dissemination and establishment of infection. Many virulent and avirulent strains have been characterized across all identified serotypes. Aragon *et al.* have thoroughly studied the differences between virulent and avirulent strains of *H. parasuis*, and identified patterns in biofilm formation, virulence markers, and presence in tissues by *H. parasuis* that may be useful for future prevention of *H. parasuis* infections. Their major finding in these comparisons demonstrates that avirulent strains of *H. parasuis* produce biofilm, whereas virulent strains do not [19]. This finding suggests that the biofilm-producing *H. parasuis* strains can remain in the upper respiratory tracts and nasal cavities of pigs, whereas the virulent strains are planktonic and can disseminate and cross the blood-membrane to cause disease. The degree of virulence for each strain also dictates its location of *H. parasuis in vivo* during the time-course of infection [34]. Avirulent strains tend to stay within the realm of the nasal turbinates or oropharynx after intranasal challenge with *H. parasuis*, whereas virulent strains will migrate to the trachea, lungs, and eventually cross the blood-membrane barrier and become systemic. While the mechanism for delocalization from the oropharynx is largely unknown, the causes for systemic infiltration of *H. parasuis* appear to involve stressor events such as weaning, transport, and recovery from prior viral or mycoplasma infection [1].

Once a *H. parasuis* infection has been established in piglets or adult swine, the symptoms displayed by the compromised animals can drastically vary. The hallmark symptom of *H.*

parasuis infection in piglets is the appearance of purple "diamond" lesions on the hock joints, indicating polyserositis [35]. Other symptoms can include neuralgia, dyspnea, acute purulent rhinitis, and pigs can become inappetant and febrile, or septic and rapidly moribund within 72 h of onset of infection [36]. Necropsy results of swine that succumbed to disease indicate the presence of fibrinous polyserositis associated with acute arthritis, which is typically called Glässer's Disease, named after the initial discoverer of the bacterium. However, many other pathologies also accompany *H. parasuis* infection. Necropsy findings can include meningitis, pericarditis, pleuritis, and pneumonia [37], especially in adult swine from specific pathogen-free or clean herds [1]. The broad spectrum of presenting symptoms and pathologies makes *H. parasuis* infection difficult to treat empirically and diagnosis is necessary for production farmers and swine veterinarians to act appropriately to protect the rest of the herd.

1.3.2 Diagnosis of Glässer's Disease

H. parasuis can be recovered from the nasal turbinates of a subclinical or infected pig by swabbing, or from collecting blood cultures from compromised animals or fluids and fibrinous tissue samples found during necropsy. The first determination a veterinarian must make is whether or not the infection was caused by a viral or bacterial agent. Usually, fibrinous exudates infer a bacterial infection, but the range of differential diagnoses makes this infection almost impossible to treat empirically. Without proper culturing techniques available at a production facility, the samples would have to be taken to a diagnostic laboratory for further evaluation.

Growth on solid agar medium is necessary for obtaining a pure culture of *H. parasuis* from the sample for an official diagnosis [35]. Many different types of agar media exist for the identification and growth of bacteria [38], and a variety of media are selected for growth to

narrow down the differential diagnoses. Fastidious bacteria such as *H. parasuis* require specific nutritional supplements within the media. *H. parasuis*, like many other Pasteurellaceae bacteria, require V factor, or nicotinamide adenine diphosphate (NAD) for growth [39]. Successful *H. parasuis* growth in a clinical diagnostic lab occurs on chocolate agar or PPLO agar supplemented with NAD [40], and usually takes 24-48 h to complete. Diagnostic labs equipped for polymerase chain reaction (PCR) may use PCR as the final step in determining the proper bacterial pathogen, differentiating other NAD-requiring bacteria from the proper diagnosis [41]. The entire procedure can take up to 72 h, from sample receipt to results, for *H. parasuis* to be properly implicated in a swine infection. However, 60-70% of the compromised herd could perish due to fatal infection by the time this determination has been made [33]. Agglutination tests have been attempted previously [42, 43], but not all isolates were able to be identified. A more rapid, efficient, and cost-effective diagnostic method is needed to prevent widespread *H. parasuis* infection in production facilities.

1.3.3 Treatment and Prevention

Because the standards of diagnosis cannot currently prevent the spread of infection within a production facility, producers and veterinarians rely on general preventative measures to avoid rampant *H. parasuis* infections. Currently ParaSail, a live-attenuated vaccine, is commercially available for protection against Glässer's Disease [8], and a course of prescribed antibiotics [44] is the standard treatment method. However, the current licensed vaccine does not protect against all serotypes, and its endotoxin content can produce dangerous side effects [45]. Moreover, the antibiotics courses used to treat infections eventually clear the bacteria from affected swine, but these drugs are expensive to administer to entire susceptible herds in farms and production

facilities. Prophylactic antibiotics can improve the overall health of a herd, but this treatment method could also put herds at risk for infections from highly virulent strains of *H. parasuis*, because strains of *H. parasuis* have emerged that are resistant to traditional β-lactam and fluoroquinolone antibiotics [46, 47]. Attempting to properly identify an effective antimicrobial treatment would take even more time in a diagnostic lab, potentially putting more animals at risk for infection. Culling of herds with diagnosed infected or carrier animals to eradicate transmission to healthy animals is easier than treatment, but also time-consuming and more costly to producers. Rapid diagnosis would lend to faster and more efficient treatment, and a safer, cost-effective vaccine that protects against infection from all serotypes would greatly improve the prevention of Glässer's Disease within a herd.

1.4 Characteristics of *H. parasuis*

1.4.1 Serotyping

To date, fifteen serotypes of *H. parasuis* have been identified worldwide through immunodiffusion [48]. Many strains of the bacterium exist within each serotype, but they can vary widely in phenotype and virulence, whereas some serotypes exhibit similar characteristics [49]. The serotype-specific antigen that caused development of unique antisera for use in this designation assay is known to be heat- and phenol- resistant, but has yet to be defined [48]. Typical antigens for Gram-negative bacteria include outer membrane proteins (OMPs), lipopolysaccharide/lipooligosaccharide (LPS/LOS), and capsular polysaccharide [50]. The most prevalent serotypes in North America are 4, 5, 13, and 14 [2]. Available vaccines are directed toward serotypes 4, 5, and 13. The CP is the serotype-specific antigen in many genera of the

Pasteurellaceae family [51], and the CP may also be responsible for *H. parasuis* type specificity [49, 52]. However, the type specificity of the CP has yet to be confirmed.

1.4.2 Molecules as Virulence Factors and/or Potential Antigenic Determinants

Strains of *H. parasuis* across all fifteen serotypes exhibit a broad range of virulence. The comparison of virulent and avirulent strains has been investigated at the genetic level. Wang *et al.* [53] proposed that the use of genetic subtraction analysis between the virulent clinical isolate SH0165 and an avirulent serotype 4 strain could highlight potential genes involved with virulence. The genes identified solely in the virulent strain encoded for export molecules, potential toxin autotransporters, and outer membrane proteins. Other global genetic studies [16, 54] identified differences between virulent and avirulent serotypes in genes responsible for outer membrane proteins (OMPs) and lipooligosaccharides (LOSs), as well as capsular polysaccharides (CPs) [18].

Proteins found on the surface of bacteria are generally responsible for localization, evasion, or harmful actions to surrounding cells [55, 56]. OmpA, found on *E. coli*, has homologues in various bacteria involved with respiratory and neural diseases. P5 and P2, two OmpA-like proteins, are found in virulent strains of *H. parasuis* [57, 58]. This is a heat-modifiable membrane protein, and in *E. coli* is upregulated in the presence of phenol [57]. OmpA and its homologous relatives act as a porin, an adhesin, and an activator of the host inflammatory and immune responses [59]. Analyses of P2 and P5 in *H. parasuis* have shown that these outer membrane proteins are immunogenic and mutants lacking these proteins can confer protection against *H. parasuis* infection in murine and swine models [56, 60]. Various groups

have theorized that subunit vaccines to these OMPs could confer protection in swine, but only partial protection against Glässer's Disease has been observed with OMP immunization [58, 61]. Moreover, the similarity of the OMPs between serotypes is too great to confer serotype specificity. The serotype-specific determinant therefore must be one of the polysaccharide molecules.

Lipopolysaccharide (LPS), or in the case of *H. parasuis* lipooligosaccharide (LOS) [50], sometimes referred to as endotoxin, is a highly reactive molecule associated with most Gram-negative bacteria [62]. Its three components, the O-antigen, core, and lipid-A vary in structure from bacterium to bacterium, and even between strains of the same species, but their functions are conserved. The O-antigen, which is the polysaccharide chain that extends outside of the cell membrane, confers antigenic identity to the LPS of a specific bacterium. Bacteria such as *E. coli* have over 160 different O-antigens, which are used as the determinants in serotyping of isolated strains [63]. The core is composed of 3-dexoy-*D-manno*-2-oculosonic acid (KDO), heptose sugars, and common hexose and hexosamine sugars, along with some amino acid residues and other non-sugar molecules, such as phosphate and ethanolamine. The core links the O-antigen to the lipid-A section of the molecule. In bacteria species that do not possess an LPS O-antigen, the core oligosaccharide becomes the antigenic determinant, and can express microheterogenicity and macroheterogenicity between strains [64, 65]. The core component also serves to maintain the structural integrity of the outer membrane of many Gram-negative species. Lipid-A, which is the predominant lipid in the outer leaflet of the outer membrane, is composed of a glucosamine disaccharide backbone to which are attached fatty acid chains of variable length and saturation. Lipid-A is the toxic component of LPS, as free Lipid-A in circulation in a host system can lead to septic shock. While it is the most volatile component of LPS or LOS, the lipid-A section of the

molecule is the most highly conserved [66]. Swine are highly sensitive to the toxic effects of LPS and LOS molecules, and the effects of the endotoxin of *H. parasuis* can have detrimental effects during infection, leading to septic shock and in some cases, death [17].

Capsular polysaccharides are another candidate for antigenic specificity. Comprised of mostly water and tightly linked sugar residues [67], usually capped with a lipid tail to anchor to the cell membrane [62], capsular polysaccharides are the serotype-specific antigen for many bacteria species, both Gram-positive and Gram-negative. The knowledge of polysaccharides encapsulating bacteria has been expanded upon since early modern bacteriology [68].

1.5 Capsular Polysaccharides

1.5.1 Gram-positive bacterial capsular polysaccharides

While most capsular polysaccharides are associated with Gram-negative bacteria, capsules occur on some Gram-positive bacteria, external to their thick peptidoglycan cell walls, and usually contain repeating chains of monosaccharides, with proteins to attach to the cell wall [69-71]. The bacterial genera with capsules include *Streptococcus*, *Bacillus*, and *Clostridium* [67]. The capsular polysaccharides of Gram-positive bacteria provide antigenic identity, in large part because they shroud other surface molecules found on the peptidoglycan wall [71]. The virulence factor most associated with capsule-proficient Gram-positive bacteria, like most capsule-proficient Gram-negative bacteria, is evasion of the host immune system by resisting complement-mediated killing due to the inability of complement molecules to bind to glycose residues, and bypassing recognition from macrophages because the glycose residues such as glucose, mannose, and sialic acid are ubiquitous on cell surfaces throughout the host [71].

1.5.2 Gram-negative bacterial capsular polysaccharides

Gram-negative capsules have been widely characterized, in part due to the numerous capsule types found in *E. coli* [72]. Four types of capsular polysaccharide loci have been identified in *E. coli*, and are used to compare sequence homology of other bacterial capsule loci. Capsule loci typically have three segments: a saccharide synthesis region responsible for the metabolism of sugar residues [73], a polysaccharide synthesis region responsible for forming polysaccharide chains and therefore conferring antigenic uniqueness to the sugar residue composition [74], and an export region, responsible for attaching the outer membrane anchor and transporting the capsule chains through the cell membrane to the surface [75]. The type 1 capsule system in *E. coli* contains high-molecular size polysaccharide chains, consisting of amino sugar residues as well as minimally acidic residues [76]. The type 1 residues also do not rely on Lipid-A for attachment to the cell membrane, but use other lipids for attachment or covalently bond to the cell surface [77]. Type 1-associated capsules, such as the capsular polysaccharide found in Enterobacteriacae species have generally conserved loci, although some genes appear to be acquired from other bacterial species [78]. Contrary to the mild acidity of the type 1 capsule, type 2 capsules contain many acidic residues, including sialic acid and KDO. Sialic acid is a component found on most host cell surfaces and when expressed on the bacterial cell surface, the bacterium can evade the immune response [79]. Also, unlike the type 1 capsule, region II of the capsule locus of type 2/3 capsules confers the serotype specificity, with regions I and III remaining conserved across serotypes [80]. Type 3 capsules are similar to type 2 capsules, with conserved regions and region II determining antigenicity. However, type 3 gene sequences have no sequence homology to type 2. Most of the Pasteurellaceae family of bacteria express type 2 or type 3 capsules [81, 82]. Type 4 capsules, the capsule type of pathogenic strains of *E. coli*,

generally consist of repeating sugars found on the LPS O-antigen, and the genes responsible for polysaccharide synthesis for both molecules are shared [83, 84]. Type 4 capsules are found in intestinal strains of *E. coli*, including both EPEC and EHEC bacteria.

Even though other bacteria express capsules that are genetically similar to *E. coli*, they do not necessarily behave like all toxin-producing Enterobacteriaceae. Toxin-producing bacteria such as *E. coli* utilize their energy to be "offensive", by adopting an aggressive approach to survival and propagation in a host. While their capsules may contribute to virulence, the capsules are not the primary method for survival. In contrast, in Gram-negative bacteria not known for exhibiting exotoxicity, such as *H. parasuis*, capsules may not only define antigenic specificity, but also contribute to bacterial virulence by promoting dissemination and recolonization, escape from phagocytosis, and protection against serum-mediated killing [85]. However, to the host's potential advantage, antibodies against capsular polysaccharides made by bacterial species that are exotoxin-deficient are protective against infection.

1.6 Capsular polysaccharide of *H. parasuis*

1.6.1 First proposed characterization of capsular polysaccharide

The serotype-specific antigen of *H. parasuis* was first isolated by Williamson and Stevenson [86], although the serotype or strain used in the study was omitted from context. Williamson and Stevenson postulated that based on work with the closely-related bacterium *H. influenzae*, the serotype-specific antigen was a polysaccharide that differed in composition across the serotypes [87]. Williamson and Stevenson used formaldehyde extraction and ethanol precipitation [88] to isolate the capsular material, broth-cultured *H. parasuis* cells were cultured on agar medium, washed, and the ethanol precipitate from the supernatants used in paper

chromatography to compare the glycose residues to those found on typical *H. influenzae* capsule types. Through colorometric assays, they determined the capsular material contained less than 1% nucleic acid and about 1% protein, and through acid and alkaline hydrolysis assays and chromatography, they determined the hexose residues present in the capsule were galactose and *N*-acetylglucosamine [86]. While the chemical analysis of this material was sound, the lack of identification of the strains of bacteria used give rise to concerns that the bacteria examined were in fact *H. parasuis*. Morozumi and Nicolet performed more definitive experiments to explore the presence of capsular polysaccharide on *H. parasuis* isolates taken from healthy pigs and pigs exhibiting symptoms typical of Glässer's Disease [52]. They demonstrated that the iridescent substance observed on the exterior of cells grown on solid agar medium, and also as a halo surrounding cells after India ink negative staining was heat-, pronase-, and phenol-resistant, all typical traits of polysaccharides. Morozumi and Nicolet [52] also used acriflavine agglutination, which indicated if the cell surface was smooth, suggestive of a capsular surface (no agglutination) or rough due to an acapsular cell membrane surface (agglutination). They also used hexadecyl trimethylammonium bromide (Cetavlon), a cationic detergent, in electrophoretic assays with capsular material from the clinical isolates, to show that cells with a negatively-charged capsular material would migrate across an agarose gel and precipitate with Cetavlon, creating a visible precipitin line in the gel. They concluded from these experiments that isolates from subclinical animals produced capsular polysaccharide, while cells found from sera and tissues during necropsy of pathological animals did not possess a capsule [52]. However, as previously discussed, this conclusion seems contradictory, as capsules from virulent bacteria create immune evasion mechanisms for the bacteria, making the bacteria more competent to survive systemically. Nevertheless, Morozumi and Nicolet proceeded with more electrophoresis

and agglutination assays of clinical isolates to attempt to classify the isolates and group then into rudimentary serotypes [49]. Morozumi and Nicolet took the same thirty-two isolates and subjected them to electrophoresis assays, as well as agglutination and immunodiffusion tests with antisera derived from rabbits immunized with *H. parasuis* bacterins from nine isolates. Based on the results of agglutination, electrophoresis migration patterns, and immunodiffusion towards one or more of the antiserum types, Morozumi and Nicolet were able to define five types of capsular polysaccharides into which twenty-six of the isolates could be grouped. Five of the isolates did not correlate with any of the designated serotypes, and even some strains that were categorized did not pass all of the assays to be included in that particular serotype. Some isolates had partial reactivity to one type of antiserum, but were grouped into a serotype because they did not react with any other antiserum. Interestingly, in this publication Morozumi and Nicolet concluded that some pathogenic strains of *H. parasuis* could be rudimently serotyped, which contradicted their original statement that only avirulent strains of *H. parasuis* were capsule-proficient.

The concept of capsular polysaccharide as the serotype-specific antigen of *H. parasuis* was not explored again until recently, even though official serotype-specific designations were outlined by Kielstein-Rapp *et al.* [48]. Olvera *et al.* echoed Morozumi and Nicolet's theories about the variability in virulence across *H. parasuis* strains, and examined various strains for their susceptibility to host immunity, specifically in regards to phagocytosis by host macrophages [33]. They performed *in vitro* assays with isolated porcine alveolar macrophages (PAMs), one of the first innate immune cells that *H. parasuis* cells would encounter when the bacteria migrate from the oropharynx. They co-incubated *H. parasuis* cells from avirulent and virulent strains with PAMs and then recovered the bacterial cells and evaluated their survival

inside and outside of the PAMs. Olvera *et al.* found that virulent strains evade phagocytosis unless either first passaged multiples times on chocolate agar, or treated with homologous antisera against the bacteria. Conversely, avirulent strains were susceptible to phagocytosis by PAMs without any treatment. Most importantly, Olvera *et al.* performed Maneval staining from *H. parasuis* cells from avirulent and virulent strains recovered after co-incubation with PAMs, and found that the phagocytosis-resistant strains produced a white, halo-like substance similar that of other known capsules. This was the first documented observation of capsule visualized on *H. parasuis* cells.

The glycose composition and structures of capsules from two serotypes were elucidated several years later by Perry *et al.* [89]. The group examined both the capsular polysaccharides and LOSs from serotype 5 Nagasaki strain, the most virulent reference strain, and serotype 15 ER-6P, an avirulent strain, by NMR-spectrometry and mass spectrometry. While the LOSs did not differ in composition or linkages, there were significant differences in the residues between the CPs of Nagasaki and ER-6P strains. In Nagasaki, the terminal sialic acid was linked to galactose-1-phosphate, whereas in ER-6P the terminal sugar was linked to *N*-acetyl-galactosamine-1-phosphate. This difference would provide enough variation in overall capsule structure between serotypes to confer antigenic specificity to each serotype.

1.6.2 The capsular polysaccharide locus of *H. parasuis*

The 14 kb capsule locus was first discovered following the sequencing of serotype 5 isolate SH0165, by Zu *et al.* [90]. However, this region was initially misidentified as the O-antigen locus for *H. parasuis* due to its homology to the O-antigen locus of *Actinobacillus pleuropneumoniae* and other Gram-negative bacteria [91]. Nonetheless, this region has been

correctly characterized as a type 1 capsule locus, which shares genes with LPS O-antigen loci [83]. The locus is segmented into two regions for synthesis, linkage, and export. Following confirmation of the CP locus in one serotype, evaluation of each of the capsular loci of the fifteen *H. parasuis* serotypes was performed by Howell *et al.* [18]. *H. parasuis* reference strains were grown on agar medium, and single colonies were picked for DNA extraction and sequencing with the Illumina HiSeq 2000 analyzer. The draft genomes were assembled using Velvet bioinformatics software, and a BLAST database was constructed using SH0165 for the reference locus, beginning at around 49 kbp on the chromosome. Each of the fifteen serotypes possessed a capsule locus, but the genes varied between serotypes. Similarities between genes and sequences exist in five distinct groups: 1, 2, 7, and 11; 9, 13, and 15; 3 and 4; 5, 6, 8, 10, and 12; 14 is a distinct group. Interestingly, the genetic groups do not correspond to the perceived virulence of each reference serotype. For example, serotype 5 is considered to be the most prevalent and virulent serotype around the globe, but serotype 10 is avirulent and only found in healthy pigs. Not only did Howell *et al.* provide information for all fifteen serotype loci, but they also predicted the function of each of the genes found in the serotype 5 SH0165 locus. Genes found directly outside of region II include NeuA, a neuraminic acid synthetase, and wzx, a putative LOS flippase. Region II genes are responsible for saccharide synthesis and linkage, including glycosyltransferases, aminotransferases, and CapD, a well-documented polysaccharide synthesis protein [92]. Region I genes are responsible for encoding export proteins, including Wza, a polysaccharide export protein, and homologues of *wzb* and *wzc* that encode for phosphatases and kinases, which assist in transport of molecules through the cell membrane [75]. The gene on the end of Region I, IscR, is a putative helix-turn-helix regulatory protein, involved in iron-sulfur regulation in *E. coli* [93]. There are currently no reports on the specific function or

role in regulation of iscR in *H. parasuis*. With the identification and characterization of the capsule locus, along with other potential virulence-associated genes, we can begin to understand the mechanisms of pathogenesis and more importantly, develop more effective prevention products against *H. parasuis* infection.

1.7 Genetic manipulation of potential virulence factors of *H. parasuis*

1.7.1 Mutagenesis strategies for *H. parasuis*

The progress of understanding the mechanisms of pathogenesis and virulence associated with *H. parasuis* come from the technological advances in molecular manipulation, not only for nucleic acid sequencing, but also in directed mutagenesis to observe the changes in targeted genes. Bisgas *et al.* created the first genetic manipulation system for *H. parasuis* [94]. They discovered the *H. parasuis* has a cyclic AMP (cAMP)-dependent natural transformation mechanism that enables the bacterium to insert DNA at the uptake signal sequence ACCGAACTC. They discovered the uptake sequence when they inserted amplified rps12, a ribosomal protein, into an *E.coli* plasmid with a gentamycin-resistance cassette using a pGEM vector, and mixed that DNA with *H. parasuis* cells on chocolate agar medium with gentamycin, and screened for cells that grew in the presence of gentamycin. Using this generated plasmid, they then introduced a mutation in the *thy* gene, cloned into this plasmid genetic material from *H. parasuis*, and introduced it into *E. coli* using a triparental mating system. They also introduced in two *Eco*RI sites on either side of the gene of interest to create a *thy-kan*^{R} fragment to insert into a suicide vector. This DNA was used in the natural transformation system. All surviving colonies were kanamycin-resistant and gentamycin-sensitive, indicating a double-crossover of the genetic material into the *H. parasuis* genome. This group also successfully used this technique when

they mutated the *fur* gene [95], a homologue to the *E. coli* iron-uptake regulatory protein, to examine previously unknown iron utilization by *H. parasuis*. However, this system may not be effective for all *H. parasuis* strains, as other groups have found some clinical isolates to be resistant to natural transformation [96].

Along with utilizing natural transformation, other groups have developed site-directed, temperature-sensitive mutagenesis systems to create knockouts in the *H. parasuis* chromosome. Wang *et al.* [97] mutated the *capD* gene in *H. parasuis* serotype 5 strain SHO165, by utilizing a temperature-sensitive plasmid, $pSHK_3^{TS}$. This plasmid was created by inserting a temperature-sensitive origin of replication in the plasmid, allowing the DNA to replicate at 30°C, but not at 41°C [98]. The sequences flanking *capD* in the *H. parasuis* SH0165 genome were amplified and inserted into the temperature-sensitive plasmid using the pSK II vector at *Eco*RI sites on either side of the kanamycin-resistance cassette. The plasmid was inserted into the *H. parasuis* cells via electroporation, plated on agar medium containing kanamycin at 30°C, and cells that grew in these conditions had *capD* mutations, confirmed by PCR of the capD region. Even though the authors reported successful deletion of *capD* and showed that the *capD* deletion mutant was attenuated in pigs and highly sensitive to complement-mediated serum killing, they did not report if there was any change in CP expression by the *capD* mutant. Positive reports of reduced CP expression would be beneficial for protective efficacy studies against *H. parasuis* infection, to observe if the mutants created were attenuated in virulence and created protection against disease.

1.8 Experimental Models for Glasser's Disease

1.8.1 *In vitro* modeling and immunoassays

For the sake of animal welfare, and cost and resources directed towards animal modeling research, most infectious disease studies begin at the cellular level. In the case of *H. parasuis*, experimental assays have been based on mimicry of the host immune system against infection. Experiments to elucidate the function and virulence of bacteria begin at the molecular level, with genetic sequencing and mutagenesis as previously discussed. The approach of "learning by breaking", in which genes are deleted and cellular function is evaluated, can be beneficial for discovering if cells can function without associated virulence factors, making the deleted molecules potential targets for drug therapies, or using the mutated bacteria as vaccine candidates. Virulence factors and other pathogenesis-associated mechanisms can be studied using *in vitro* assays, such as assessing growth efficiency in environmental conditions, co-culturing with other bacteria or host immune cells, or immunoassays including antigen reactivity in ELISAs, labeling for microscopy, and serum-sensitivity tests. Bacteria must rely on the host system for survival and replication, and while ideal conditions exist, bacteria must adapt or die. Determining and then manipulating the optimal growth conditions that mimic the host is beneficial for understanding virulence. In the early discovery and classification of *H. parasuis*, NAD and not hemin requirement for growth was noted by screening hundreds of *Haemophilus* isolates [15]. Through this screening the bacterium was given its own taxonomic recognition. Other experiments have demonstrated that *H. parasuis* favors aerobic environments and oxygenated conditions [99], and utilizes oxygen as an intermediary for metabolic pathways, but oxygen is not required for growth, as NADH molecules are recycled for cellular functionality. Microarrays performed by Melnikow *et al.* [100] were done in growth environments that mimic

physiological scenarios in the swine host, including iron-deficiency, elevated temperature, and acidic blood conditions that mirror physiologic stressors. RNA was extracted from these cells at given time-points and used in microarray analyses to determine gene regulation during these conditions. Seventy-five genes were identified whose regulation changed during these conditions, and genetic analysis and comparison to other related bacteria showed that some of these genes were involved in virulence. *H. parasuis* was identified because of co-culturing experiments with other bacteria and viruses [13], and valuable information regarding its ability to evade the host immune system have been previously discussed [33]. In addition, Zhang *et al.* determined that even though *H. parasuis* cells evaded phagocytosis, they still elicited an inflammatory response to infection, potentially causing damage to surrounding healthy tissue [59]. To determine the cause, they purified P2, an OMP, treated PAMs with the protein, and measured cytokine mRNA levels in the presence or absence of P2. They found that IL-1, IL-6, and IL-8 increased in the presence of P2 in a dose-dependent manner, demonstrating that OMPs caused a pro-inflammatory immune response. Immunoassays can be valuable for looking for virulence-associated molecules, or demonstrating reactivity to potential antigens. The fifteen serotypes were initially designated based on immunodiffusion of cellular extracts towards antisera against whole cells of *H. parasuis* [48]. Immunoassays, such as dotblots or ELISA, can be used to determine if a molecule on the surface of bacteria is recognized by the host immune system. In the case of the LOS of *H. parasuis* [101], colony blots were developed and incubated with immune sera to known LOS molecules from various serotypes to see if any antigenic variation was present. No variation was observed in the LOS molecules, and it was concluded that the LOS probably did not lend to the antigenic variability of *H. parasuis*. Cells and cellular molecules can also be identified using immuno-histochemistry. Bello-Orti *et al.* [34] used whole

cell immune sera derived from rabbits to identify where cells of *H. parasuis* were located on tissue sections from the nasal turbinates, trachea, and lungs to determine where the bacteria were located at different time-points after infection, and based on their virulence. They determined that avirulent strains tended to stay close to the oropharynx throughout the time-course of infection, while virulent strains migrated towards the lungs and crossed the blood-membrane barrier as time progressed. While *in vitro* assays are an effective and powerful launching point to explore virulence and possible protection models, animals are necessary to generate immune sera for these tests, and must be used to provide a platform for experiments that are not entirely restrained by laboratory environmental controls.

1.8.2 The mouse model for Glässer's Disease and its inherent issues

Animal models are an effective means to understand how disease progresses and to test drug therapies and attenuated pathogens to observe what happens in real time, *in vivo*. Mice are often considered an effective model system for a wide range of pathogens, due to the depth of understanding of their anatomy and physiology, ease in genetic manipulation, having control over a living system, and abundance for statistical significance when observing results of experiments. The murine model has been used in *H. parasuis* experiments to generate antibodies, as well as to test the protective efficacy of mutated bacterial strains when challenged with the parent strain. Morozumi developed the first mouse model for *H. parasuis* challenge experiments [102], when he inoculated mice intraperitoneally with upwards of 2×10^9 CFU of various *H. parasuis* strains. Death only occurred in mice challenged with the greatest concentration of bacteria, and he was able to recover *H. parasuis* cells from the brain and lung tissues of the moribund mice, even though no tissue lesions were observed on any of the tissues during

necropsy. He attributed the death of these mice to septicemia, which is the result of the immune system's response to an overload of endotoxin, causing a cytokine storm that leads to perfusion of blood into tissues throughout the host [103]. Even though these preliminary findings formed an argument against the mouse as an effective model for Glässer's Disease, many other groups have used mice in their experiments. Tadjine *et al.* developed monoclonal antibodies against agar-grown whole cells of *H. parasuis* serotype 4 strain SW124 [104]. The antibodies were used in ELISA and Western Blot experiments with purified OMPs, LOS, and whole cells of serotype 4 strain SW124, and they observed that the antisera reacted strongly with both the OMPs and LOS of *H. parasuis*. However, in further investigations using the antisera, one of the antibodies, 4D5, did not react with any cell surface antigen examined by transmission electron microscopy (TEM), and reacted with only 30% of clinical isolates in indirect agglutination studies, unless the cells were sonicated. This suggests that the antigenic epitope used to develop the antiserum is either not constitutively present on the cell surface, or could be shrouded by another molecule during infection. After it was determined that mice could develop an antibody response against *H. parasuis* antigens, several groups from China have attempted to use the mouse model for infection studies. Yuan *et al.* [105] developed recombinant outer membrane proteins to use as vaccine candidates in mice. The three OMPs were used to immunize mice, along with a negative control. They determined that the antibodies developed against the OMPs were mostly IgG_2, and cytokine evaluation showed that a Th_2 response was the prevalent immune response to these antigens. However, when the mice were challenged, they were given exceptionally high doses of *H. parasuis* from serotypes 4 and 5, upwards of $7x10^9$ CFU/mouse. Even though the dosages were very high, very few mice immunized with the OMPs succumbed to disease, and no bacteria were recovered from the surviving mice at necropsy. However, negating these findings was their

absence of proof that bacteria were able to be recovered from the mice that did succumb to disease, especially the negative control mice. Even the histopathology from this protection study did not show any bacteria in the tissues from the negative controls. Perhaps the BALB/c mice, the same strain used previously by Morozumi, were moribund due to septicemia because of the high levels of endotoxin present in the bloodstream, and not from the establishment of Glässer's Disease in these mice. Moreover, other groups have attempted to use mice in OMP subunit vaccine studies [106-108], but the "established" lethal dose for the same inbred strain of mice that Morozumi and Yuan utilized ranged from 1.2×10^9 to 5×10^9. These groups reported that control and mock-vaccinated mice succumbed to "disease" within two days, but little evidence of an established *H. parasuis* infection was offered in any of these publications. Interestingly enough, groups outside of China have abandoned the mouse model for Glässer's Disease, citing that the mice would clear the bacteria within 24 h of challenge with lower titers of *H. parasuis,* but the threshold between clearance and disease due to endotoxemia was too narrow to establish an effective model (V. Aragon and S. Brockmeier, personal communication). The reasons for inconsistency for establishing Glässer's Disease in mice could lie with the fact that *H. parasuis* is a host-specific pathogen, and requires a swine host for survival and replication. The source of iron (host transferrin) between pigs and mice differ, and host specificity is likely due to iron sequestration [109] and colonization factors. Pigs remain the preferential model for investigation of Glässer's Disease for experimental treatments.

1.8.3 Advances in understanding Glässer's Disease in the Swine model

The pig model has proved effective for observing and understanding Glässer's Disease, in both controlled experimental herds as well as field herds. The first documented observations

regarding Glässer's Disease occurred when the bacterium was discovered [13], when *H. parasuis* was isolated from pigs exhibiting similar pneumonia symptoms, and similar lesions and pathologies were observed in necropsies. T.W. Little was able to establish Glasser's Disease in piglets by intranasal, intratracheal, and intraperitoneal inoculation, following Koch's postulates, with *H. parasuis* recovered from clinically moribund swine [110]. The discovery of snout-snout contact to establish colonization in piglets was determined by Amano *et al.* [111], when the group took seronegative piglets and inoculated them intranasally with bacteria from *H. parasuis* serotypes 4 or 5, and then exposed the inoculated pigs to seronegative piglets. Swabs and necropsies of all of the piglets showed that *H. parasuis* had established a presence the oropharynx of each pig that had been exposed to *H. parasuis*, directly or through snout contact, whereas isolated seronegative pigs in the same facility had no *H. parasuis* colonization. Vahle *et al.* [35] determined that the health status of the pigs influenced *H. parasuis* colonization and dissemination, where piglets challenged with *H. parasuis* were monitored for the presence of systemic bacteria, and tissue evaluation was performed at necropsy. Infected piglets were found to have compromised cilia-mucosal tracts, which could have influenced migration of the bacteria into the lungs, and then across the blood-membrane barrier to establish Glässer's Disease. Other groups determined that the serotype of *H. parasuis* influences the chances of systemic infection. After the serotypes had been designated, it was shown by Blackall *et al.* that only certain serotypes could establish infection in the pig, and some serotypes were only found in the oropharynxes of subclinical, asymptomatic pigs [112]. Recently, Bello-Orti *et al.* observed the pathogenic differences between virulent and avirulent strains of *H. parasuis* [19, 34] in infected piglets over several time-points post-infection. They were able to characterize the bacterial strains based on location in the piglet post-infection, creating an effective model of *H. parasuis*

virulence in the host. They determined that avirulent strains tend to colonize the nasal cavities and upper respiratory tracts of animals without establishing infection, but virulent strains could disseminate and cause disease. The strains found in the oropharynx generated biofilms, but strains found systemically were planktonic. Further evaluation of changes within the bacteria as well as the host will be valuable in further developing the understanding of the pathogenesis of Glässer's Disease.

While research has been performed to understand *H. parasuis* infection, other pig models have been established to evaluate control mechanisms to prevent Glässer's Disease. Minats *et al.* [113] showed that cross- protection was possible with pigs vaccinated with whole cell bacterins from virulent strains of *H. parasuis*, but incomplete protection was afforded from bacterins generated from avirulent strains. Minats concluded that the antigenicity of various serotypes of *H. parasuis* was linked to virulence, but no molecule for antigenic specificity was identified. Other bacterin studies have been performed with similar results [114, 115]. Bacterins are one example of a killed-cell vaccine useful in immunizing animals, depleted of their ability to replicate or cause virulence in the host, but still available to create a potent immune response from both B and T cells. Nonetheless, the LOS can still be present on these cells, causing harmful side effects in piglets and the factors responsible for protective immunity still remain unknown. If the antigens responsible for a potent host immune response could be identified, safe and cost-effective subunit vaccines, along with diagnostic tools to identify and evaluate *H. parasuis* could be produced to efficiently control and potentially eradicate Glässer's Disease in production herds.

1.9 Concluding Remarks

My dissertation research is based on the hypothesis that the capsular polysaccharide of *H. parasuis* is the serotype-specific antigen, contributes to virulence and pathogenesis of Glässer's Disease, and can be used in diagnostics and vaccines to identify and control infection in swine. To verify this, I outlined three objectives to explore the role and potential use of CP in *H. parasuis*: 1) Purify and characterize the CP from various serotypes of *H. parasuis*, and evaluate its expression in various environmental conditions; 2) Develop antibodies against these CPs and use them not only in the characterization of CP, but also in a latex agglutination assay to detect *H. parasuis* infection in swine and to assist with proper typing of various *H. parasuis* clinical isolates; 3) Create a CP-protein complex that can generate a robust antibody response against CP in piglets, and evaluate the protective efficacy of a CP subunit vaccine against Glässer's Disease. These objectives all evaluate the role of CP in *H. parasuis*, both from a pathogenesis perspective and as strategy for control of disease.

The CP of *H. parasuis* is a strong candidate for the serotype-specific antigen of each serotype. When the serotypes were designated using immunodiffusion, the antigen was determined to be heat- and phenol-resistant [48]. Most proteins are not heat stable due to denaturation, let alone phenol stable due to the solubility of proteins in a less polar solvent than water. Therefore, outer membrane proteins are not a likely candidate. Moreover, even if OMPs were resistant to heat and phenol, they would be shrouded by the bacterium's capsule during pathogenesis, and therefore would not be presented to the host immune cells for recognition. The LOS could be another potential candidate, but the LOS of *H. parasuis* lacks O-antigen, and studies regarding the LOS of this bacterium have shown that there is minimal variation in LOS

composition and structure between serotypes [89, 104]. Therefore, capsule, another polysaccharide molecule, could be the antigenic determinant.

Although *H. parasuis* is capable of producing CP, as demonstrated by the presence of CP loci in all fifteen serotypes [18], CP has not been shown to be present on all *H. parasuis* cells [52]. This could be due to variability in synthesis and expression of the CP material. Most Pasteurellaceae bacteria produce capsules constitutively [10, 116], possibly in varying quantities across serotypes and environmental conditions, but perhaps these bacteria only survive in conditions that require the production of CP. Since *H. parasuis* lives subclinically in the oropharynx of pigs, and then disseminates due to an event likely caused by stress, the CP may be not be expressed on the cell surface until needed for virulence. If cells colonizing the oropharynx create a biofilm [19], it would not be energy-efficient for the cell to make CP at that time. Moreover, the reasoning behind ineffective protection studies in pigs could be due to the presence or absence of CP on the cells used in infection. The conditions under which CP is expressed must be evaluated.

The CP from *H. parasuis,* if determined to confer antigenic identity to each serotype, could be used in a latex agglutination assay to diagnose and serotype cells recovered from pigs with Glässer's Disease. This type of assay has been used with *A. pleuropneumoniae*, whose antigen has been confirmed as CP [117]. If the latex agglutination assay is effective for the rapid diagnosis of Glässer's Disease, it could replace the days-long procedure to confirm the presence of *H. parasuis*, saving many pigs from succumbing to infection. Moreover, many labs have reported strains of *H. parasuis* as untypeable, [3, 6, 29, 49, 112], and perhaps the reason for the inability behind the lack of properly serotyping these clinical isolates has to do with the presence of mulitple serotypes of *H. parasuis* or multiple species of CP-producing bacteria at the time of

isolation, or poor sensitivity and/or specificity of available typing methods If CP is produced but expressed in varying quantities, a sensitive assay based on antisera against CP could provide answers to the inability to serotype all *H. parasuis* strains.

This research utilizes various animal models, including mice, rabbits, and pigs. While the use of mice as a model of Glässer's Disease remains controversial, mice are an efficient source of antibody and cytokine production against specific antigens. Specific IgG antisera against pig antibodies are not readily commercially available at this time, nor are the ones that are available sufficient in discerning IgG isotypes (C. Loving, personal communication), whereas murine reagents are widely available and very accurate. Rabbits are generally used for larger-scale antibody generation. Polysaccharides are generally not efficient at generating an antibody response, especially if glycose residues such as sialic acid, found ubiquitously on all cell types, exist in the CP. Freund's adjuvant, which assists in stimulating the primary immune response will assist with boosting titers against the specific CP antigen [117]. Pigs are the ultimate model for testing vaccine candidates and their protective efficacy against Glässer's Disease. The LDs_{50} of virulent serotypes 4 and 5 have been established in the pig model [16], as well as procedures for bacterial challenge and health monitoring. CPs will be conjugated to carrier proteins, since CPs are not good at generating a robust immune response on their own [118]. While antibodies are effective at protecting against exotoxin-deficient, CP-proficient bacterial infection [85], a humoral response is necessary, but not sufficient, at clearing bacteria from the host. Previous protection studies with pigs have shown that successful protection against Glässer's Disease involved both Th_1 and Th_2 responses [105, 107]. It would be beneficial to understand the host response to vaccination with CP, to establish a standard for protection and eradication of *H. parasuis* in swine populations.

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Summary of attempts to replicate Anne Hyman's dissertation results

Following submission of a manuscript on the type specificity of the capsular polysaccharide of the swine pathogen *Haemophilus parasuis* to the journal PLoS One there was a request by reviewers for improved quality of photographs and other data, necessitating a postdoc in my lab and I to redo some experiments. First of all, new capsular polysaccharide was purified from a serotype 5 strain. There are 15 serotypes of *H. parasuis*, which we hypothesized were due to antigenic differences in the capsular polysaccharides of each serotype. Unfortunately, the experiments concerning the antiserum that Anne Hyman had generated were unable to be repeated, and were entirely out of line with the results described.

Anne immunized rabbits with what was supposed to be a purified polysaccharide of *H. parasuis.* She told me the rabbit was responding to the immunizations and making a response. Anne showed me ELISA data indicating that the titer of antiserum to the capsule was >1:10,000.

Anne's claims based on the antiserum she prepared:
1, the capsule is the serotype specific antigen of *H. parasuis*. This means that antibodies to the capsule react predominately, though not necessarily exclusively, with only that one serotype.

Anne's results presented to PLoS One:

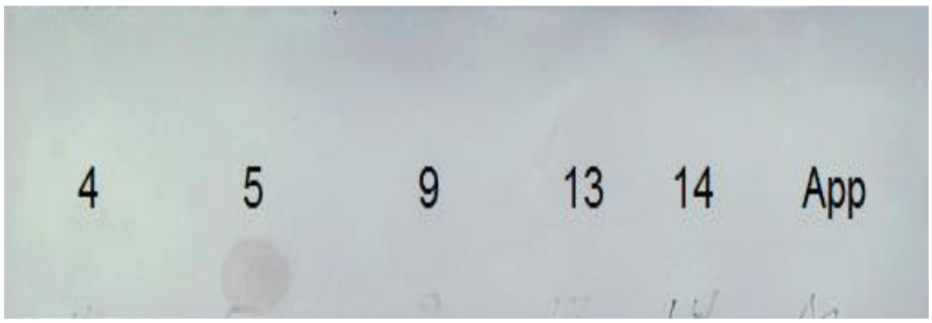


Fig. 3. Reactivity of *H. parasuis* serotypes with antiserum to *H. parasuis* serotype 5 CP. All serotypes were grown in broth, and 2 µl of each sample was dotted onto nitrocellulose and incubated with anti-CP 5 serum. App- *A. pleuropneumoniae.*

This figure shows that antiserum to the capsule of *H. parasuis* serotype 5 reacted with only the capsule of serotype 5, and not to the capsules of serotypes 4, 9, 13, 14 or *A. pleuropneumoniae* (App). Numbers refer to the *H. parasuis* serotype.

Our repeat of this experiment:

Our repeat shows that Anne's serum reacts with the capsule of all serotypes tested, as well as to whole cells of *A. pleuropneumoniae* and a very unrelated organism, *Francisella tularensis.*

2, the capsule is expressed by cells grown in broth, but not on agar plates.
Anne's results presented to PLoS One:

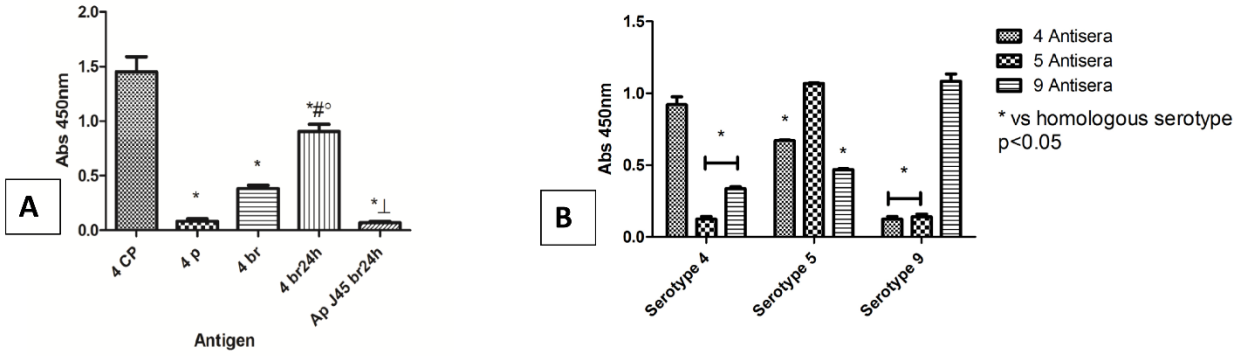
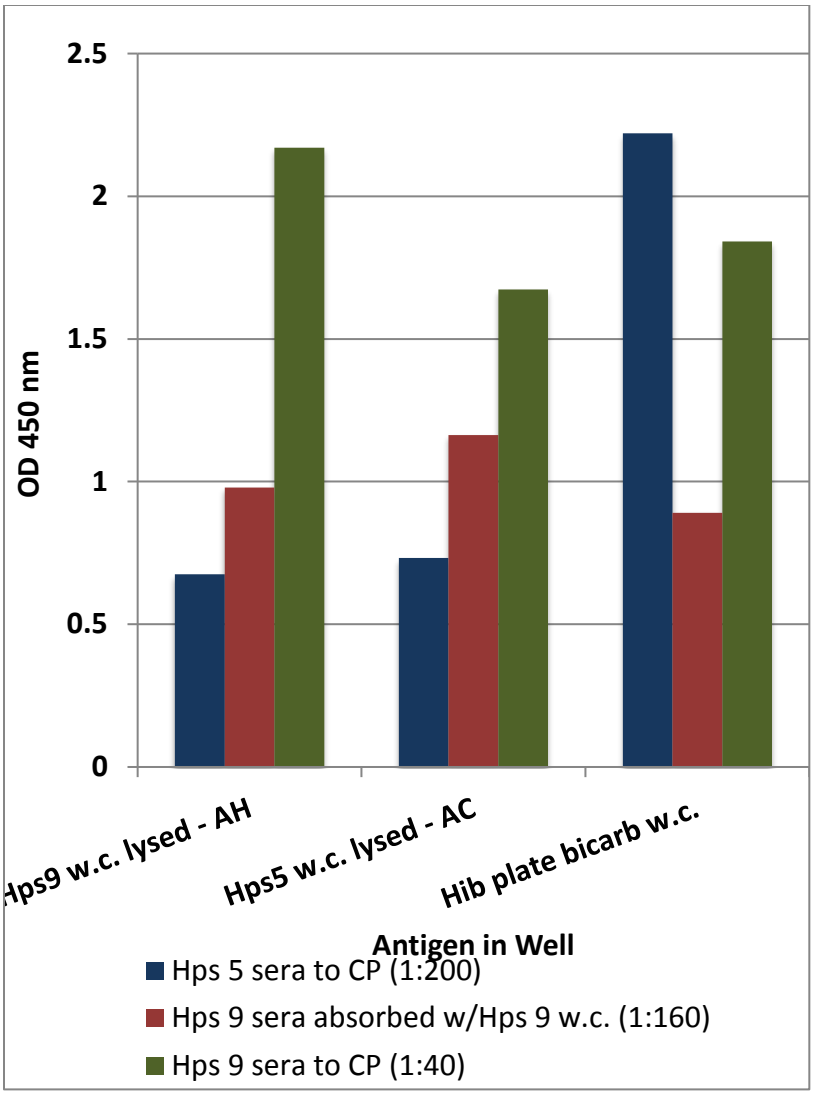


Fig. 4. Homologous and heterologous reactivity of antiserum to *H. parasuis* purified CP or whole cells by ELISA. Purified CP, or bacteria grown on agar (p), in broth to mid-log phase (br), or for 24 h (br24h) were used as antigens with homologous or heterologous antisera. A, reactivity of antiserum to serotype 4 CP with homologous CP, with agar-grown cells (p), with bacteria grown in broth for 12 hours (br) or for 24 h (br24h). *, indicates a significant difference compared to reactivity to serotype 4 CP ($p < 0.01$), #, indicates a significant difference compared to reactivity with serotype 4 cells (p) ($p < 0.01$), °, indicates a significant difference with reactivity to serotype 4 cells (br) ($p < 0.05$), ⊥, indicates a significant difference compared to reactivity with serotype 4 cells (br24h) ($p < 0.01$). B, reactivity of antisera to CPs 4, 5, or 9 with homologous and heterologous CPs. * indicates a significant difference in reactivity to heterologous antisera in comparison to reactivity with homologous antiserum (All significance = $p<0.05$).

This figure shows that antiserum to serotype 4 reacts with purified capsule, and to cells grown in broth, but not to cells grown on agar plates or to *A. pleuropneumoniae* grown in broth (A). B shows that antiserum to the same antigenic serotype reacts significantly greater with the capsule of that serotype than to capsules of other serotypes.

Our results:



These results do support Anne's results in that at least the antiserum to serotype 9 capsule reacts most strongly to serotype 9. However, antiserum to serotype 9 capsule also reacted more straongly with serotype 5 capsule than antiserum to serotype 5, and all the sera reacted strongly with *H. influenzae* type b, an unrelated species.

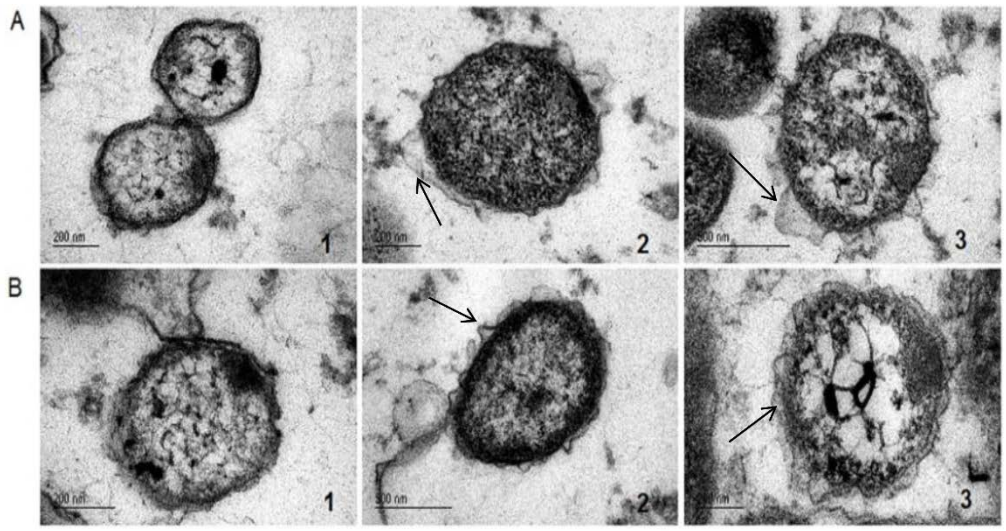
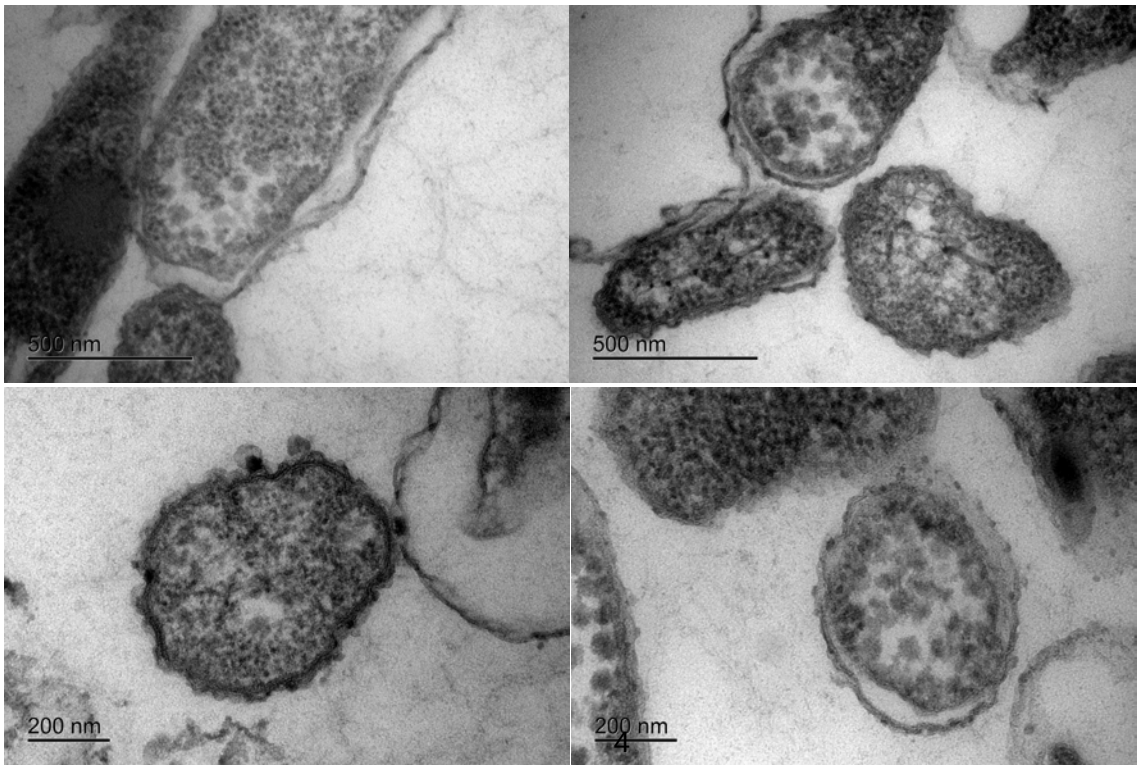
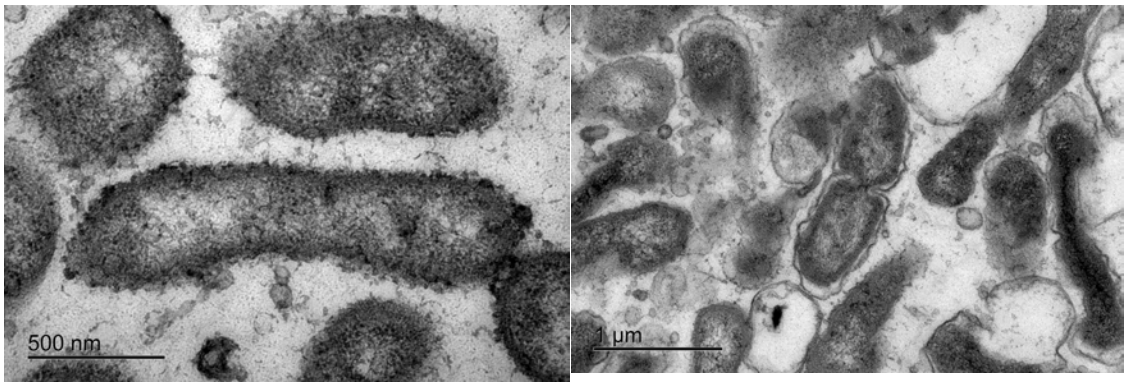


Fig. 6. Immuno-electron microscopy of *H. parasuis* for CP expression. *H. parasuis* serotypes 4 (A) or 5 (B) were grown on agar (1), or in broth to mid-log phase (2), or in broth for 24 h (3). The bacteria were washed and incubated with homologous antibody to CP. The bacteria were stained with Ruthenium red, fixed, dehydrated, stained with uranyl acetate, and examined by transmission electron microscopy. Bacteria grown on agar did not exhibit the ruffled, halo-like CP structure observed around broth-grown cells (arrows).

Another figure showing by electron microscopy that capsule is only expressed on the surface of cells grown in broth. The reviewers questions if the material pointed out is actually capsule.

Our results:





Top row- *H. influenzae* type b grown in broth
Middle row- *H. parasuis* type 5 grown in broth
Bottom row- *H. parasuis* type 5 grown on agar plates.

I see no clear evidence of a capsule on any of the bacteria tested. Because capsules consist of primarily water they are very difficult to see by electron microscopy, which is done in a vacuum.

3, bicarbonate increases capsule production on cells grown in broth and on agar plates

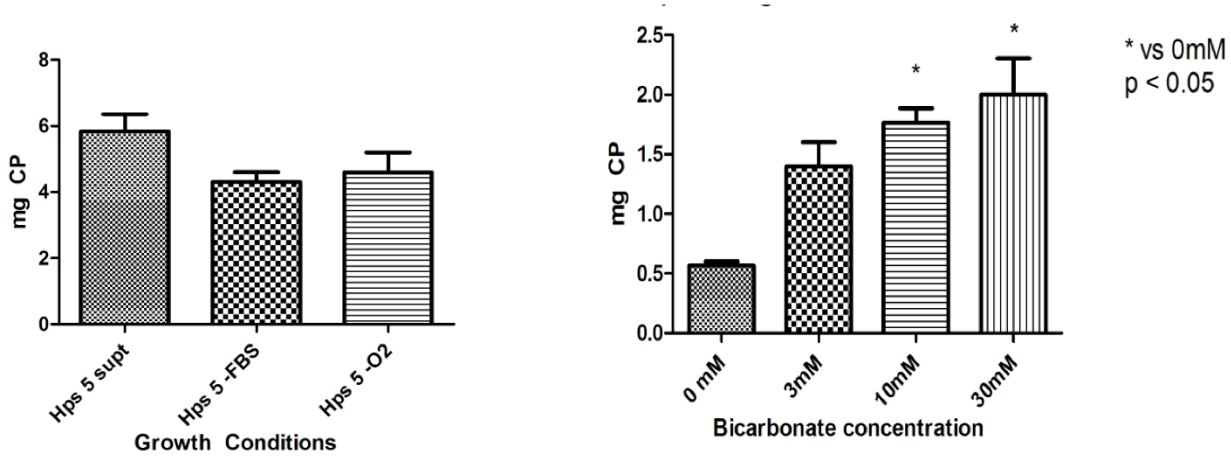
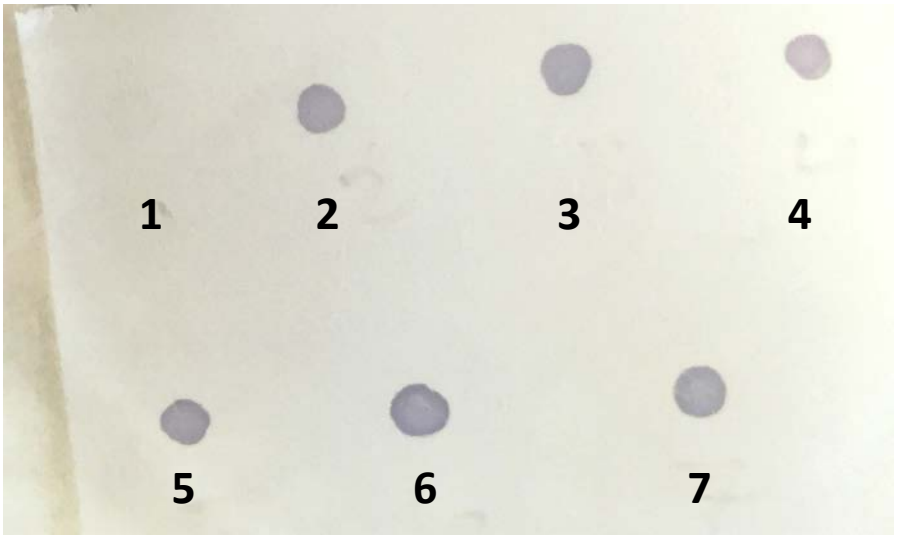


Fig. 9. Growth supplements affecting CP production. (A) *H. parasuis* (Hps) was grown in PPLO$^+$ broth containing 5% fetal bovine serum (FBS), NAD, and 1% glucose (supt); PPLO+ medium lacking FBS (-FBS); PPLO$^+$ medium supplemented with 10% Oxyrase to remove oxygen (-O2); or (B) PPLO$^+$ supplemented with 3-30 mM sodium bicarbonate. * indicates significantly more CP produced ($p < 0.05$ for 10 mM bicarbonate supplementation, $p<0.01$ for 30 mM bicarbonate supplementation) than the 0 mM bicarbonate control.

This figure shows that if the bacteria are grown in medium containing sodium bicarbonate in broth that capsule production is significantly increased.

Our results:



1. type 5 - Pure CP (capsular polysaccharide)
2. type 5 – Pl (cells grown on plates)
3. type 5 – Pl – bicarb (cells grown on plates with bicarbonate)
4. type 5 – Br (cells grown in broth medium)
5. type 5 – Br – bicarb (cells grown in broth medium with bicarbonate)
6. type 5 - Pl – lysed (cells that have been grown on plates and lysed)
7. type 5 – Pl – bicarb – lysed (cells that have been grown on plates with bicarbonate and lysed)

We see no evidence that bicarbonate enhances production or release of the capsule.

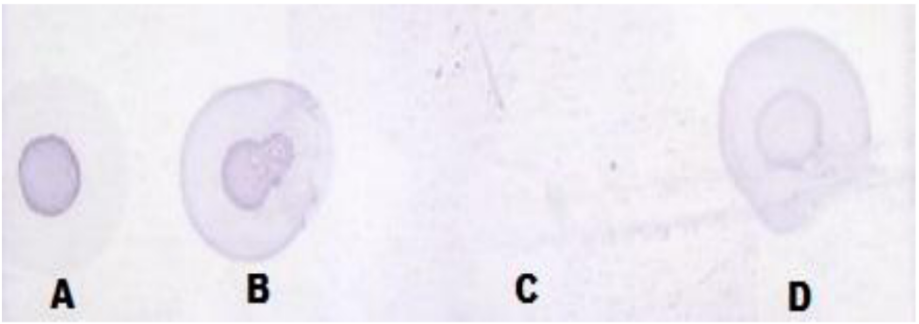


Fig 10. Immuno-blot of *H. parasuis* serotype 5 grown on agar with or without bicarbonate supplementation. A, purified serotype 5 CP; B, broth-grown serotype 5 cells; C, agar-grown serotype 5 cells on PPLO$^+$; D, agar-grown serotype 5 cells on PPLO$^+$ supplemented with 30 mM bicarbonate.

This figure also shows that if the bacteria are grown on agar plates with or without bicarbonate, that capsule can now be detected on cells grown with bicarbonate, but not without bicarbonate. Anne's blots were on nitrocellulose.

Our results:

Nitrocellulose. 1:250 anti-Hps5 CP sera

Hps5 w.c. plate Hps5 w.c. plat w/bicarb Hps5 w.c.–broth Hib w.c. broth Hps5

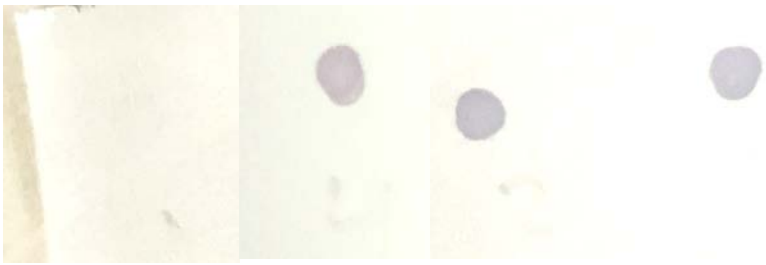
CP +Charged Nylon. 1: 250 anti-Hps5 CP sera

Hps5 w.c. plate Hps5 w.c. plat w/bicarb Hps5 w.c.–broth Hib w.c. broth Hps5 CP

Definitions: Hps5-*H. parasuis* serotype 5
w.c.-whole cells
plate-cells grown on agar plates
broth-cells grown in broth medium
CP-purified capsular polysaccharide
w/bicarb-sodium bicarbonate added to the growth medium

In our repeat experiment, new highly purified type 5 CP did not react with the antiserum on nitrocellulose, but did react on charged nylon paper. Cells of another encapsulated bacterium, *Haemophilus influenzae* type b, also reacted very strongly with the antiserum. *H. parasuis* cells grown on agar without bicarbonate reacted as well with the antiserum as cells grown with bicarbonate.

Also:



A **B** **C** **D**

An exact replica of Anne's blot on nitrocellulose showing that the Hp5 capsule does not appear, but all whole cells tested did, including cells off plates.

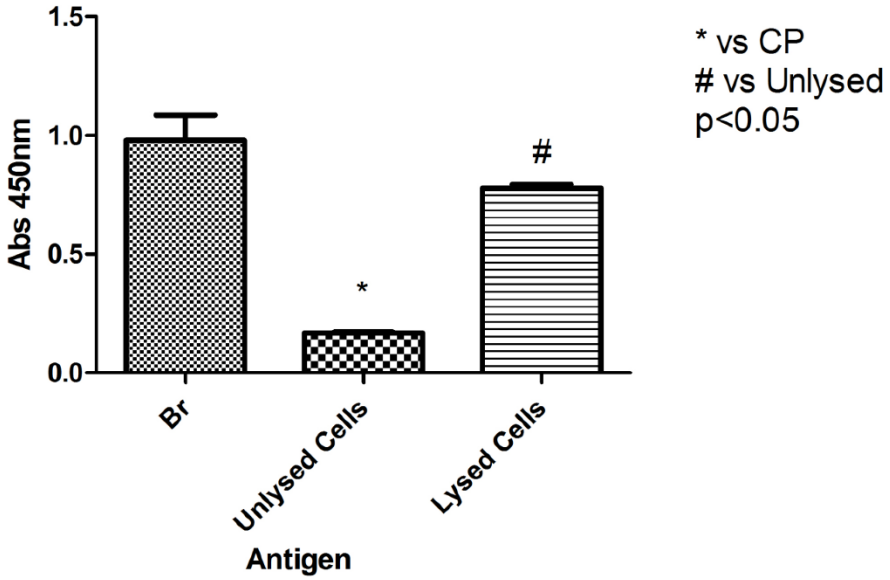
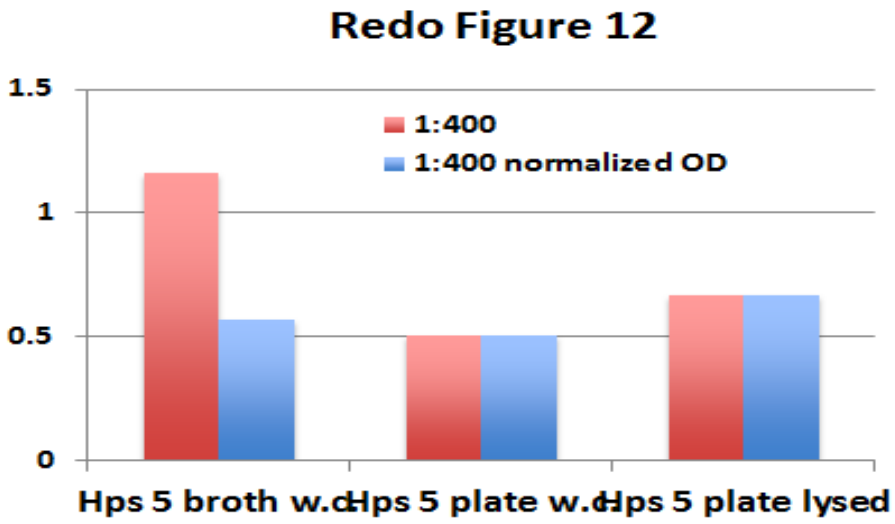


Fig. 12: Production of CP by agar-grown cells. The amount of CP from *H. parasuis* serotype 5 grown in broth (Br) was compared by ELISA to the material extracted from bacteria grown on agar that were lysed or left intact. There was significantly more CP produced by broth-grown bacteria than unlysed agar-grown bacteria (*, $p < 0.05$) and significantly more CP produced by lysed, agar-grown bacteria than unlysed agar-grown bacteria (#, $p < 0.05$). There was no significant difference between CP production by broth-grown bacteria or by lysed agar-grown bacteria ($p > 0.05$).

This figure shows that if the bacteria grown on agar plates are lysed, that capsule can be detected with the antiserum to the capsule by ELISA. This indicates that the capsule is made, but not exported when the bacteria are grown on agar plates, and that it is exported when grown in broth. This result would explain why the bacteria need to be lysed by autoclaving in order to be typed.

Our results:



There was slightly more capsule present from lysed cells, but this is not unusual. The difference in the amount of capsule from lysed cells and unlysed was not significant.

All of the data shown involved the use of the antiserum Anne made to “purified" *H. parasuis* capsule. Prior work by a commercial laboratory indicated that the capsule is not immunogenic in rabbits.

In summary, my conclusion after extensive investigation and many repeated experiments over the past year (many others are not shown here, but were supplied separately), is that Anne’s data could not be replicated as reported in the dissertation and that the amount of sialic acid on the capsule is greater than we thought and makes the capsule non-immunogenic.

In other experiments carried out by colleagues in pigs in Iowa, pigs immunized with the capsule conjugated to an immunogenic protein also did not make antibodies to the capsule.

Statement prepared by:
Thomas J. Inzana, Ph.D., D(A.B.M.M.), F(A.A.M.)
Tyler J. and Frances F. Young Chair in Bacteriology
Section Head, Clinical Microbiology
University Research Integrity Officer
Virginia-Maryland College of Veterinary Medicine
Virginia Tech-Carilion School of Medicine
Life Sciences 1, 970 Washington St., SW
Virginia Tech, Blacksburg, VA 24061

Chapter 2

Expression and Type-Specificity of the Capsular Polysaccharide of *Haemophilus parasuis*

Anne C. Michalenka Hyman[1]
Cristina De Castro[2]
Antonio Molinaro[2]
Thomas J. Inzana[1,3]

[1]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia, United States of America

[2]Department of Organic Chemistry and Biochemistry University of Naples "Federico II", Italy

[3]Virginia Tech Carilion School of Medicine, Roanoke, Virginia, United States of America

2.1 Abstract

Haemophilus parasuis is a Gram-negative bacterium responsible for Glässer's Disease in pigs. However, little is known regarding the bacterium's antigenic specificity or virulence factors. Our goal was to isolate the *H. parasuis* capsular polysaccharide, and determine its role in serotype specificity and virulence. Capsules were purified from several *H. parasuis* serotypes using enzyme digestion, phenol extraction, and ultracentrifugation. The glycose profiles of *H. parasuis* capsules contained similar sugars, but greatly varied in quantity. Capsule was not isolated from agar-grown bacteria unless the cells were lysed or grown with medium supplemented with bicarbonate. Bicarbonate also augmented capsule quantity in broth-grown bacteria, demonstrating that external factors may regulate capsule production and expression. Capsule was only visualized on broth-grown cells incubated with homologous anti-capsule serum by immunofluorescence and transmission electron microscopy. Although capsules from some serotypes cross-reacted to a limited degree in sensitive immunoassays, capsules from each serotype were nonetheless immunodominant. In contrast the lipooligosaccharides from all serotypes tested cross-reacted equally with antiserum to each serotype, confirming that the capsule and not the lipooligosaccharide, was responsible for serotype specificity. Capsule-deficient *H. parasuis* was highly susceptible to serum-mediated killing, but encapsulated bacteria were serum-resistant unless homologous anti-capsule serum was added, indicating capsule played an important role in protecting the bacteria from normal host defense mechanisms. Some genes responsible for capsule export and synthesis, including the putative transcriptional regulator *iscR*, were upregulated in broth-grown and bicarbonate-treated *H. parasuis* cells compared to bacteria grown on agar medium, demonstrating that external stimuli triggered and

also regulated expression of the capsule locus. Regulation of capsule expression may explain why serotyping and virulence is highly variable in *H. parasuis*, and is discussed.

2.2 Introduction

Haemophilus parasuis is a pleomorphic Gram-negative bacterium in the family Pasteurellaceae and is responsible for Glässer's Disease (polyserositis) in piglets and pneumonia in adult pigs. *H. parasuis* infects pigs worldwide, and is most commonly seen in animals from high-health or specific pathogen-free herds, or as a co- or secondary pathogen following previous viral or mycoplasma infection. Infected pigs may develop polyserositis and arthritis, meningitis, pneumonia, or sepsis, and the disease can often be fatal [1]. Symptoms are generally observed in piglets 3 weeks to 4 months of age due to the stresses of weaning, relocation, or prior infection [2]. Vaccines (bacterins or a live-attenuated strain) are available, but current vaccines do not protect against all serotypes [3, 4] and the endotoxin present in whole cells can cause dangerous systemic side effects [5]. Proper diagnosis for effective treatment is time-consuming, due to the fastidious nature of the bacterium. In the time needed to grow *H. parasuis* from infected sites for diagnosis by conventional means or by PCR [6], *H. parasuis* can devastate up to sixty percent of a herd [7]. Therefore, a safer, cost-effective *H. parasuis* vaccine and a rapid screening test for detection of *H. parasuis* serotypes are needed to maintain optimum health in swine herds.

Fifteen *H. parasuis* serotypes have been identified globally. The most prevalent serotypes in North America are 4, 5, 13, and 14 [1]. Available vaccines are directed toward serotypes 4, 5, and 13. However, the molecular determinants responsible for protection and serotype specificity are undefined, although the serotype-specific antigens are known to be heat-, pronase-, and phenol-resistant [8, 9]. The most common bacterial antigens resistant to these treatments that are

associated with type specificity are capsular polysaccharide (CP) and lipopolysaccharide (LPS), or in the case of *H. parasuis* lipooligosaccharide (LOS) [10]. The CP is the serotype-specific antigen in many genera of the Pasteurellaceae family [11], and the CP may also be responsible for *H. parasuis* type specificity [12, 13]. However, the type specificity of the CP has yet to be confirmed. The CP may also contribute to bacterial virulence by promoting resistance to phagocytosis and complement-mediated killing [14]. As a result, antibodies to the CP would be expected to be opsonic and fix bactericidal complement, which would promote clearance of the bacteria. Therefore, the CP could be efficacious as a component in vaccines and in diagnostic assays [15, 16]. In this work we report for the first time that the capsule is the type-specific antigen of *H. parasuis* and that its expression is regulated by growth in broth and bicarbonate supplementation, which appears to be due to export of the capsule. Furthermore, we present preliminary evidence that the *H. parasuis* CP contributes to resistance to host defenses.

2.3 Materials and Methods

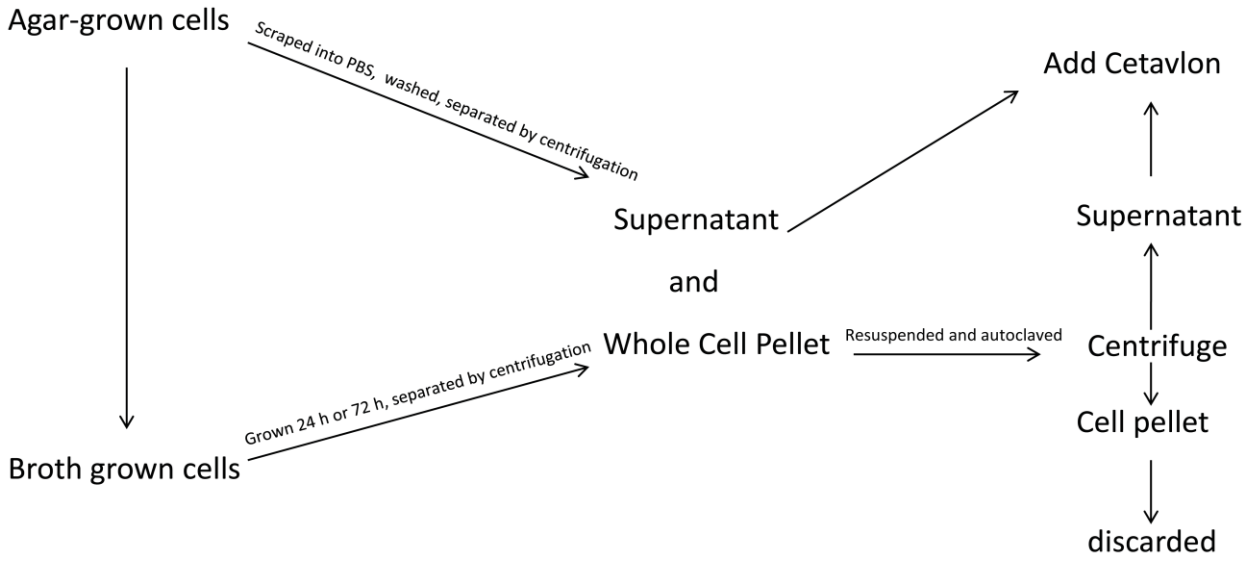
2.3.1 Bacterial strains, media, and growth conditions

H. parasuis reference serotypes were acquired from Dr. Linda Zeller at the Iowa State University School of Veterinary Medicine. *Actinobacillus pleuropneumoniae* strain J45 [17] was used as a control. Bacteria were grown on chocolate agar plates (Remel, Lenexa, KS) or Pleuropneumoniae-like organism (PPLO)-agar plates (BD, Franklin Lakes, NJ) supplemented with 100 µg/ml of nicotinamide adenine dinucleotide (NAD) (Acros, Geel, Belgium) and incubated for 24 h at 37 °C, or in PPLO broth supplemented with 100 µg/ml of NAD, 5% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), and 1% glucose (Sigma, St. Louis, MO) ($PPLO^{+}$).

Bacterial strains were grown on chocolate agar or PPLO$^+$ agar for about 48 h, or grown in 20 ml to 2 L of PPLO$^+$ broth shaken at 37 °C to mid-log phase, late log phase (24 h), or stationery phase (72 h). The number of bacterial cells/ml of culture was determined spectrophotometrically and confirmed by viable plate count. Aliquots of all cultures were reserved for immunoassays. Bacteria were grown on solid agar medium as above or medium supplemented with 30 mM bicarbonate (Sigma). In some cases bacteria were inoculated to PPLO broth without glucose or fetal bovine serum, or with Oxyrase at a concentration of 10% (v/v) (Oxyrase Inc., Mansfield, OH). Broth cultures were incubated as above.

2.3.2 CP purification

CP was purified by modification of previous procedures [17]. The bacteria were scraped from solid agar medium into PBS, or grown in PPLO$^+$ broth to log or stationary phases, and the cells were sedimented by centrifugation at 10,000 x *g* at 4 °C for 15 minutes (all subsequent centrifugation steps were performed at 4 °C for 15 minutes unless noted). The pellet was resuspended in 50 ml of distilled water, heated at 121 °C for 30 minutes in an autoclave, the mixture subjected to centrifugation as above, and the supernatant saved to isolate any CP in and on the bacteria (S1 Fig.).



S1 Fig. Growth and treatment of *H. parasuis* for CP isolation. *H. parasuis* was grown on agar medium for inoculation to broth or scraped directly into PBS, and the bacteria sedimented by centrifugation. The cell pellets from the agar and from the broth were resuspended and autoclaved to lyse the bacteria, the lysate centrifuged, and the supernatant recovered following centrifugation. The pellets were discarded and 10 mM Cetavlon was added to all supernatants.

Cetyltrimethylammonium bromide (Cetavlon; Sigma) was added to the supernatants from both the $PPLO^{+}$ broth and the agar-grown cells at a final concentration of 10 mM and held at 4 °C overnight. Following centrifugation, the pellets were resuspended in 50 ml of 50 mM Trizma base, 50 mM $CaCl_2$, 50 mM $MgCl_2$, and 0.05% sodium azide (Sigma). The suspensions were treated with 100 U of Riboshredder (EpiCentre, Madison, WI) and 100 U of DNAse (Sigma) for 1 h each at 37 °C, followed by 50 μg of Proteinase-K (Sigma), and the incubation continued overnight at 56 °C. The mixture was then extracted with 45% aqueous phenol (Fisher Scientific, Fair Lawn, NJ) for 30 min at room temperature and the mixture centrifuged. The aqueous phase was removed without disturbing the interface, distilled water was added to the phenol phase, and the extraction procedure was repeated until no interface material was present. The aqueous

phases were pooled and dialyzed against repeated changes of distilled water until there was no detectable phenol odor.

Any remaining LOS in the samples was removed by ultracentrifugation at 41,000 x *g* for 4 h at 4 °C. The supernatant was lyophilized, resuspended in a minimum amount of distilled water and eluted through Sephacryl S-400 in a (39 x 1)-cm column at a flow rate of 1 cm^3/10 min. Carbohydrate-positive fractions that eluted in the void volume were pooled, dialyzed, and lyophilized. Total carbohydrate was determined by a reduced volume phenol-sulfuric acid assay [18]. Protein concentrations were determined by BCA assay (ThermoPierce, Fair Lawn, NJ). If necessary, repeated enzyme digestions and a repurification scheme were added [19].

2.3.3 LOS purification

LOS was extracted using a previously reported method [20]. The LOS pellets were resuspended in 50 µl HPLC water and stored at -20°C for electrophoresis and immunoblotting.

2.3.4 RNA Extraction and qRT-PCR

Bacteria were grown either on agar and scraped into sterile PBS or in $PPLO^+$ broth and treated with RNAprotect (Qiagen, Valencia, CA). Bacterial cells stored at -20 °C were lysed with 1 mg/ml lysozyme in diethylpyrocarbonate water (Sigma), and the RNA extracted with the RNEasy kit (Qiagen). RNA concentrations were determined on a Thermo Scientific Nanodrop 1000 (Fisher), and samples were stored in RNAse-free water at -80 °C. cDNA was made from the RNA using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). The cDNA was used in quantitative real-time PCR (qRT-PCR) to measure mRNA expression of the genes in the CP locus of *H. parasuis* serotype 5. qRT-PCR primers were designed using IDT PrimerQuest

(IDT, Coralville, IA) using sequences with GenBank accession numbers KC795336-KC795346, KC795348-KC795350 [21].

2.3.5 Gel electrophoresis

CP electrophoretic profiles were determined by electrophoresis of 20 μg of CP in 25% acrylamide native gels [22] and stained with Alcian Blue and Silver stain (Bio-Rad, Hercules) [23]. LOS electrophoretic profiles were determined by electrophoresis of 2 μg of LOS, as previously described [24], and stained with ammoniacal silver [25].

2.3.6 CP Composition

Purified CPs from serotypes 4, 9, and 13 were analyzed for composition. Monosaccharides were identified as acetylated O-methyl glycoside derivatives. Methanolysis was performed using 2 M HCl/MeOH at 85 °C for 24 h. The sugars were acetylated with acetic anhydride in pyridine at 85 °C for 30 min. The sugar residues and their absolute configuration were determined by gas-liquid chromatography (GLC) and GLC-mass spectrometry (GC-MS), as described [26].

2.3.7 Antiserum

This study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, National Research Council of the National Academies). The protocol was approved by the Institute Animal Care and Use Committee of Virginia Tech (Protocol Number: 12-073-CVM).

Rabbits were immunized subcutaneously with 100 μg of CP from serotypes 4, 5, and 9 in sterile PBS mixed 1:1 with Freund's Complete adjuvant (Sigma) as described [16]. Blood samples were obtained prior to immunization to establish baseline titers to CP, and then subsequently every two weeks to monitor antibody titers. Animals were boosted with the same concentration of the homologous CP subcutaneously in Freund's Incomplete adjuvant 4 weeks post-inoculation. Booster immunizations of the same concentration of CP were given intravenously in saline 4 weeks after the second immunization, and then every two weeks until the titer reached ≥1:10,000 by enzyme-linked immunosorbent assay (ELISA).

Antisera to *H. parasuis* whole cells of serotypes 4, 5, and 9 were acquired from Drs. Susan Brockmeier and Crystal Loving at the National Animal Disease Center in Ames, Iowa.

2.3.8 Immunoblotting

Two μl of purified CP or LOS (1 mg/ml), or *H. parasuis* (10^8 cells/ml) were dotted onto a nitrocellulose membrane (Whatman, Dassel, Germany) and dried at 37 °C. Membranes were blocked with 5% non-fat dry milk (NFDM) in PBS, rinsed in PBS containing 0.05% Tween 20 (Fisher) (PBST), and incubated with a 1:500 dilution of either anti-CP serum (for reactivity to CP or whole cells) or whole-cell anti-*H. parasuis* serum (for LOS) in 5% NFDM in PBST. After 1 h incubation at room temperature the membranes were washed with PBST, and a 1:5000 dilution of horse radish peroxidase (HRP)-tagged anti-rabbit or anti-swine IgG (Jackson Labs, Bar Harbor, ME) was added and incubated at room temperature for 1 h. The membranes were then washed in PBST and the blots developed with 3,3',5,5'-tetramethylbenzidine (TMB)-peroxidase substrate (Kirkgaard-Perry Laboratories, Gaithersburg, MD).

2.3.9 ELISA

Ten µg/ml of purified CP or 10^8 cells/ml of *H. parasuis* in PBS containing 2 mM $MgCl_2$ (PBS-M) were added to wells of high-binding 96-well plates (Immulon 4HBX, Fair Lawn), and incubated at 37 °C for 1 h. The plates were washed with PBST, and then blocked using 2% NFDM in PBST at 37 °C for 1 h. The plates were washed with PBST, and incubated with serial dilutions of antiserum in 2% NFDM in PBST at 37 °C for 1h. The plates were washed as above and HRP-tagged anti-rabbit IgG (Jackson Labs, Bar Harbor, ME) diluted 1:5000 in PBST was added and incubated at 37 °C for 1 h. The plates were washed with PBST and color was developed with TMB-peroxidase (ThermoPierce, Fair Lawn, NJ). The reaction was stopped with 1 M H_2SO_4 and the absorbance read at 450 nm.

2.3.10 Bactericidal assay

The bactericidal assay was carried out as previously described [27], with minor modifications. Briefly, the bacteria were grown to mid-log phase (10^9 CFU/ml, determined spectrophotometrically) or for 24 h, washed in PBS, and resuspended to 10^4 CFU/ml in PCM. Normal swine serum, 20% precolostral calf serum (PCS) as a complement source, or 10-50% antiserum supplemented with PCS was added to the bacteria on ice and mixed. Twenty µl of the mixture was immediately spread onto 2 quadrants of a chocolate agar plate (0 min), and the remaining mixtures were incubated for 60 min at 37 °C. After incubation 20 µl of the mixture was spread onto the remaining two quadrants of the same plates (60 min). The chocolate plates were incubated for 24 h, and percent survival was determined by dividing the number of colonies at 60 min by the number of colonies at 0 min and multiplying by 100.

2.3.11 Transmission Electron Microscopy (TEM)

TEM for analysis of CP was carried out by modification of a previously described method [28]. The bacteria were grown to mid-log phase in $PPLO^{+}$ broth or for 24 h, washed in PBS, and resuspended to 10^4 CFU/ml in PCM. The bacteria were incubated with antiserum diluted 1:20 for 1 h at 37 °C, and any unbound antisera was removed by centrifugation for 10 min at 5000 x *g*, and then fixed in cacodylate buffer supplemented with 5% glutaraldehyde and 0.15% Ruthenium red (Sigma) for 2 h at room temperature. The bacteria were fixed in 4% agar, washed 5 times in cacodylate buffer, and post-fixed for 2 h with 2% osmium tetroxide. The agar blocks were washed as above, and the samples were dehydrated using a graded series of acetone washes. All the solutions used in processing the specimen, from the wash after glutaraldehyde fixation to dehydration with the 70% acetone solution, contained 0.05% (wt/vol) Ruthenium red. Samples were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin. Thin sections were post-stained with uranyl acetate and lead citrate and examined with a JEOL JEM 1400 Transmission Electron Microscope at an accelerating voltage of 80 kV.

2.3.12 Immunofluorescence Microscopy

Bacteria grown to mid-log phase or for 24 h were washed and resuspended in PCM. The bacteria were diluted to about 10^3 CFU/ml, spread onto borosilicate glass slides, and dried. The slides were fixed in 10% formalin/PBS for 10 min, quenched with 100 mM glycine for 10 min, washed in PBS for 10 min, and dried. A 1:20 dilution of CP antiserum (or PCM as a control) was placed onto each slide, and incubated in a sealed, humid chamber for 1 h at 37 °C. The slides were placed in PBST for 10 min, allowed to dry, and a 1:20 dilution of fluorescein isothiocyanate (FITC)-anti-rabbit or mouse IgG (Fisher) was added. The slides were incubated in

a sealed, humid chamber for 1 h at 37 °C, then placed in PBST for 10 min, dried, and sealed with 50% glycerol under a coverslip. The slides were examined with an Olympus IX81 fluorescence microscope at 100x magnification with either bright field or FITC fluorescence light emission.

2.3.13 Data processing and statistical analyses

Student's t-test and two-way ANOVA with Bonferroni post-test calculations were determined using InStat GraphPad Prism v. 5.02 to determine statistical significance. Histograms and survival curves were also generated with GraphPad Prism.

2.4 Results

2.4.1 Chemical and electrophoretic analysis

Extracts from cells grown on agar or broth, and culture supernatants were purified for polysaccharide as described in methods. Culture supernatants from mid-log phase broth cultures contained 30.9 μg of carbohydrate/g cells (wet weight), and 58.7 μg of carbohydrate/g cells from 24 h cultures. In contrast, extracts from whole cells grown in broth contained 14.3 μg of carbohydrate/g cells, whereas extracts from whole cells grown on $PPLO^{+}$ agar contained 0 μg of carbohydrate (data not shown). The electrophoretic profile of each sample is shown in Fig. 1. Samples purified from broth-grown cells produced an electrophoretic profile similar to that of the CP of *A. pleuropneumoniae* strain J45, whereas samples extracted from agar-grown cells contained no material that could be stained with Alcian Blue and silver. The polysaccharides purified from whole cells grown in broth and from broth culture supernatants produced similar profiles that varied primarily in quantity.

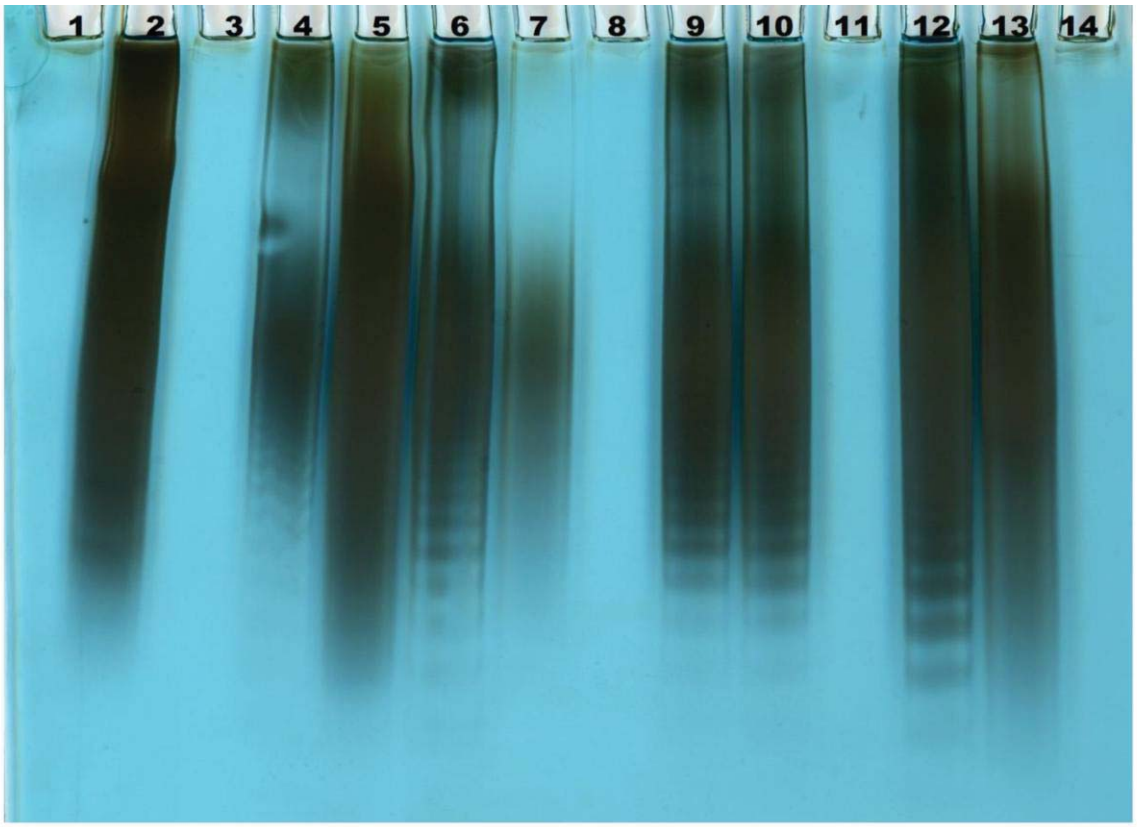


Fig. 1. Electrophoretic profiles of CPs from *H. parasuis* serotypes grown in broth or on agar. Culture supernatants or bacterial cells were extracted and purified for CP, electrophoresed through a 25% polyacrylamide gel, and stained with alcian blue and silver for polysaccharide. Lanes and extracts from: 1, blank; 2, *A. pleuropneumoniae* strain J45 (p); 3, *H. parasuis* serotype 4 (p); 4, *H. parasuis serotype* 4 (cs); 5, *H. parasuis* serotype 4 (s); 6, *H. parasuis* serotype 5 (cs); 7, *H. parasuis* serotype 5 (s); 8, *H. parasuis* serotype 5 (p); 9, *H. parasuis* serotype 9 (cs); 10, *H. parasuis* serotype 9 (s); 11, *H. parasuis* serotype 9 (p); 12, *H. parasuis* serotype 13 (cs); 13, *H. parasuis* serotype 13 (s); 14, *H. parasuis* serotype 13 (p). p, polysaccharide isolated from whole cells grown on agar; cs, polysaccharide isolated from whole cells grown in broth; s, polysaccharide isolated from broth culture supernatant.

Analysis by GC-MS indicated that the CPs of serotypes 4, 9, and 13 contained glucose, galactose, mannose, N-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid, but different sugars predominated in different serotypes. The CP of serotype 4 contained primarily galactose together with comparable amounts of mannose, glucose, glucosamine, and galactosamine, the CP of serotype 9 contained predominately galactose together with mannose and *N*-acetylglucosamine, while galactosamine and glucose appeared as minor components, and the CP of serotype 13 contained predominately glucose, while galactose and *N*-acetylglucosamine were in comparable abundance (Fig. 2). Thus, the structures of these serotypes are likely to be distinct or composed of different polysaccharides in varying amounts.

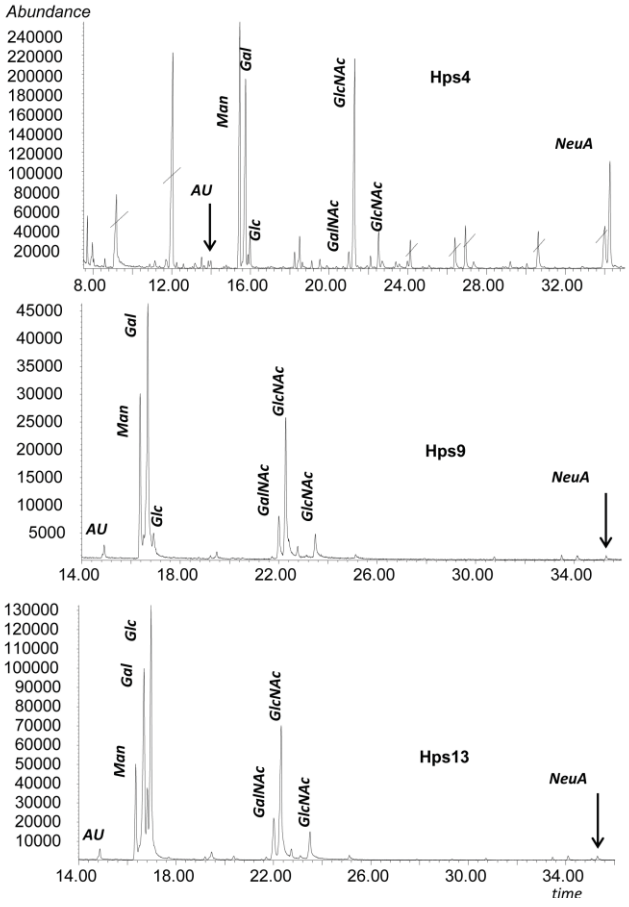


Fig. 2. GC-MS analysis of glycoses from *H. parasuis* serotypes 4, 9, and 13 CPs. Sugars identified: Glc (glucose), Man (mannose), Gal (galactose), GalNAc (*N*-acetylgalactosamine),

GlcNAc (*N*-acetylglucosamine), NeuA (neuraminic [sialic] acid), AU is an unidentified uronic acid. Peaks crossed with a dotted line are contaminants.

2.4.2 Antigenic specificity of *H. parasuis* CP

Broth-grown cultures of *H. parasuis* serotypes 4, 5, 9, 13, and 14 were blotted onto nitrocellulose membranes and tested for antigenic reactivity with antiserum to *H. parasuis* serotype 5 purified CP (Fig. 3). Antibodies to the serotype 5 CP reacted only with the homologous serotype by immunoblotting. Antiserum to the purified CPs of serotypes 4 and 9 also reacted only with the homologous serotype (data not shown).

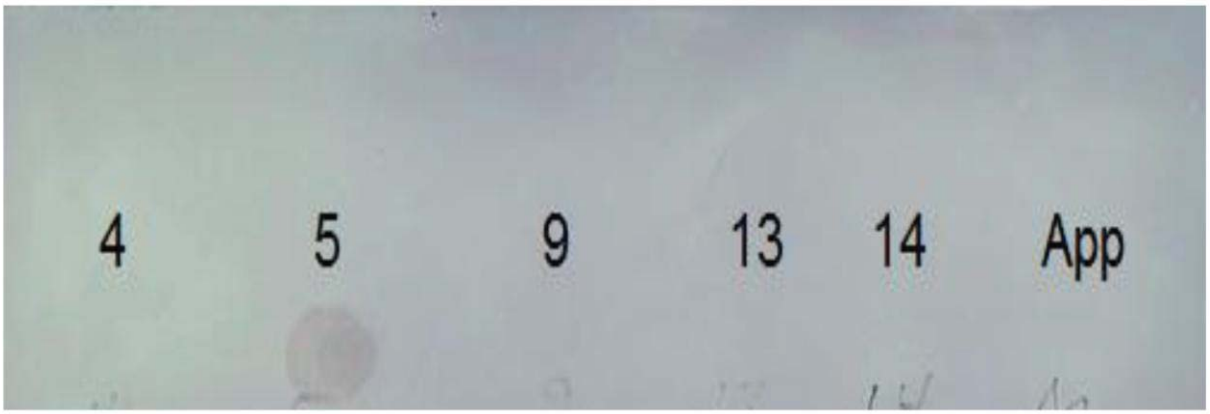


Fig. 3. Reactivity of *H. parasuis* serotypes with antiserum to *H. parasuis* serotype 5 CP. All serotypes were grown in broth, and 2 µl of each sample was dotted onto nitrocellulose and incubated with anti-CP 5 serum. App, *A. pleuropneumoniae.*

H. parasuis cells grown on agar or in broth, and purified CP were tested for serotype specificity by ELISA. The reactivity of antiserum to serotype 4 CP was greatest with the purified homologous CP, but reactivity also occurred with homologous whole cells grown in broth to mid-log phase or for 24 h; reactivity was significantly greater with bacteria grown for 24 h ($p<0.05$). *H. parasuis* grown on agar was no more reactive with the homologous antiserum to CP than with broth-grown *A. pleuropneumoniae* strain J45 cells (Fig. 4A). Similar results were

observed with antiserum to serotypes 5 and 9 CPs (data not shown). However, when broth-grown cells were tested for reactivity with heterologous antisera, some reactivity was noted, but was significantly less than with the homologous serotype, $p<0.05$ (Fig. 4B).

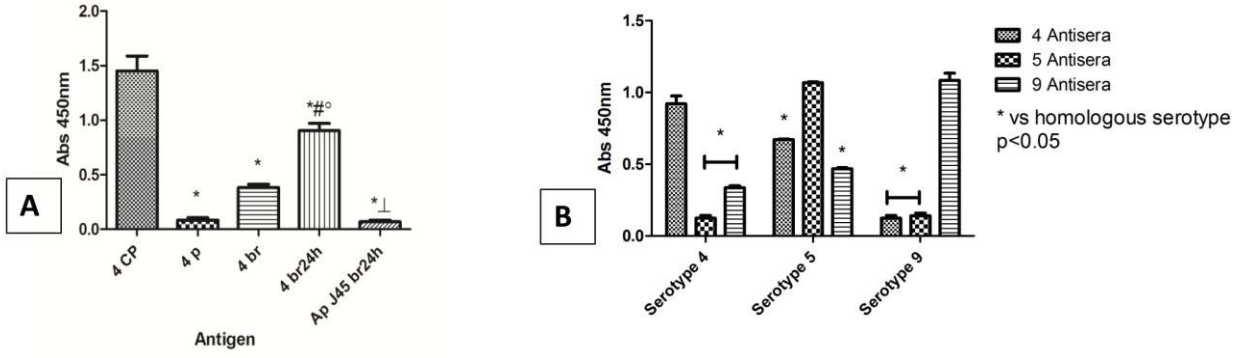


Fig. 4. Homologous and heterologous reactivity of antiserum to *H. parasuis* purified CP or whole cells by ELISA. Purified CP, or bacteria grown on agar (p), in broth to mid-log phase (br), or for 24 h (br24h) were used as antigens with homologous or heterologous antisera. A, reactivity of antiserum to serotype 4 CP with homologous CP, with agar-grown cells (p), with bacteria grown in broth for 12 hours (br) or for 24 h (br24h). *, indicates a significant difference compared to reactivity to serotype 4 CP ($p < 0.01$), #, indicates a significant difference compared to reactivity with serotype 4 cells (p) ($p < 0.01$), °, indicates a significant difference with reactivity to serotype 4 cells (br) ($p < 0.05$), ⊥, indicates a significant difference compared to reactivity with serotype 4 cells (br24h) ($p < 0.01$). B, reactivity of antisera to CPs 4, 5, or 9 with homologous and heterologous CPs. * indicates a significant difference in reactivity to heterologous antisera in comparison to reactivity with homologous antiserum (All significance = $p<0.05$).

2.4.3 Surface expression of CP

*H. parasuis s*erotype 5 was grown on agar or in broth to mid-log phase or for 24 h. The bacteria were fixed, incubated with antiserum or PBS, then with FITC-labeled anti-rabbit IgG, and examined for fluorescence by fluorescence microscopy (Fig. 5). Bacteria grown on agar or grown in broth and incubated with PBS did not exhibit any fluorescence. However, the surface of bacteria grown in broth and incubated with homologous antserum to serotype 5 CP fluorescenced, indicating expression of the CP on the surface of the cells grown in broth, but not on agar. Similar experiments with antiserum to CPs of serotypes 4 and 9 with broth-grown and agar-grown cells generated identical results (not shown).

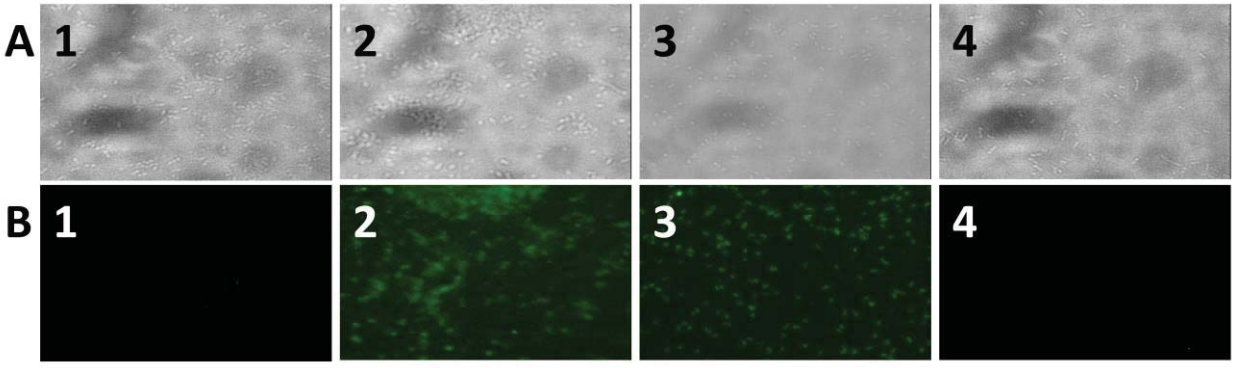


Fig. 5. Expression of CP on *H. parasuis* by immunofluorescence. *H. parasuis* serotype 5 was grown on agar medium (1), in broth to mid-log phase (2), or in broth for 24 h (3, 4). The bacteria were incubated with (1-3) or without (4) rabbit antiserum to serotype 5 CP followed by FITC-labeled anti-rabbit IgG. A, bright-field microscopy; B, fluorescence microscopy.

Serotypes 4 and 5 cells grown on agar, or in broth for 4 h or 24 h, were washed and incubated with homologous antiserum. The cells were then stained with Ruthenium red, fixed, dehydrated, and mounted before staining with uranyl acetate and visualized by TEM. (Fig. 6).

The cells grown in broth exhibited a ruffled "halo" surrounding the cells (arrows), consistent with a CP [28]. In contrast, agar-grown bacteria were devoid of any such surface material.

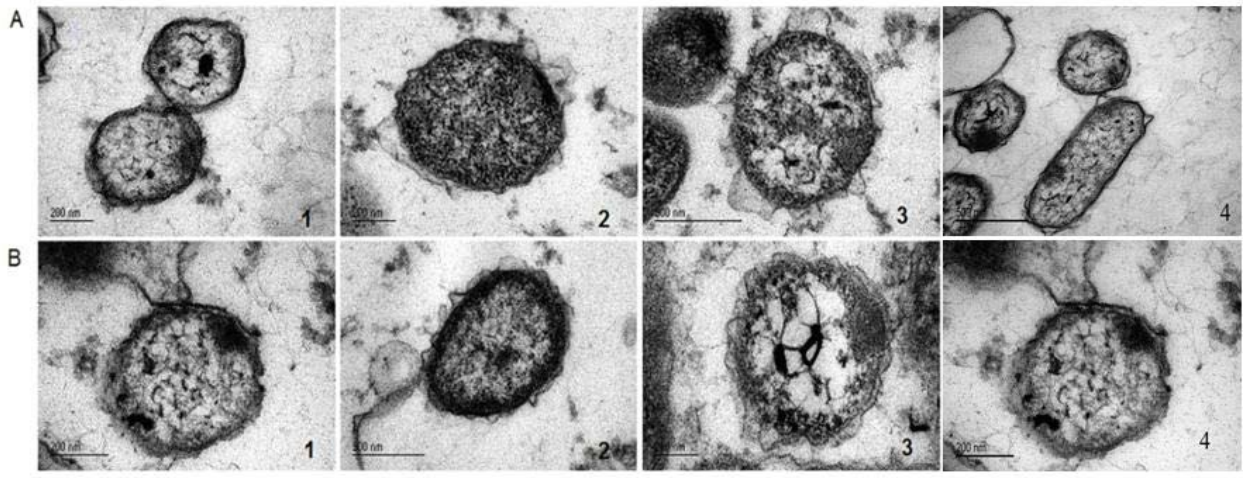


Figure 6. Immuno-EM with Ruthenium red staining of *H. parasuis* cells grown on agar (1) or in broth (2, 3). Cells from serotypes 4 (A) and 5 (B) were grown on agar (1), or in broth to mid-log phase (2), or for 24h (3), washed, and incubated with (1-3) or without (4) homologous antibody to CP. Cells were then stained with Ruthenium red, fixed, dehydrated, and mounted before staining with uranyl acetate and visualized by TEM. Cells grown on agar did not exhibit the ruffled, halo-like CP structure observed around broth-grown cells (arrows).

2.4.4 *H. parasuis* LOS electrophoretic and antigenic analyses

LOS was extracted from all 15 serotypes grown on agar medium by hot phenol extraction, followed by centrifugation and ethanol precipitation. Each LOS was spotted onto a nitrocellulose membrane and blotted with whole cell antiserum to serotypes 4, 5, or 9 (LOSs from serotypes 3, 4, 5, and 9 shown in Fig. 7A). The electrophoretic profile of each LOS was also examined by gel electrophoresis and silver staining (Fig. 7B; LOS from serotypes 3, 4, 5,

and 9 only shown). Unlike the CPs of these serotypes, the LOSs from serotypes 3, 4, 5, and 9 all reacted strongly with each antiserum, including to the heterogeneous serotypes. In addition, the LOS of each *H. parasuis* serotype displayed little to no microheterogeneity, unlike related family members, such as *Histophilus somni* and *Haemophilus influenzae* [29, 30].

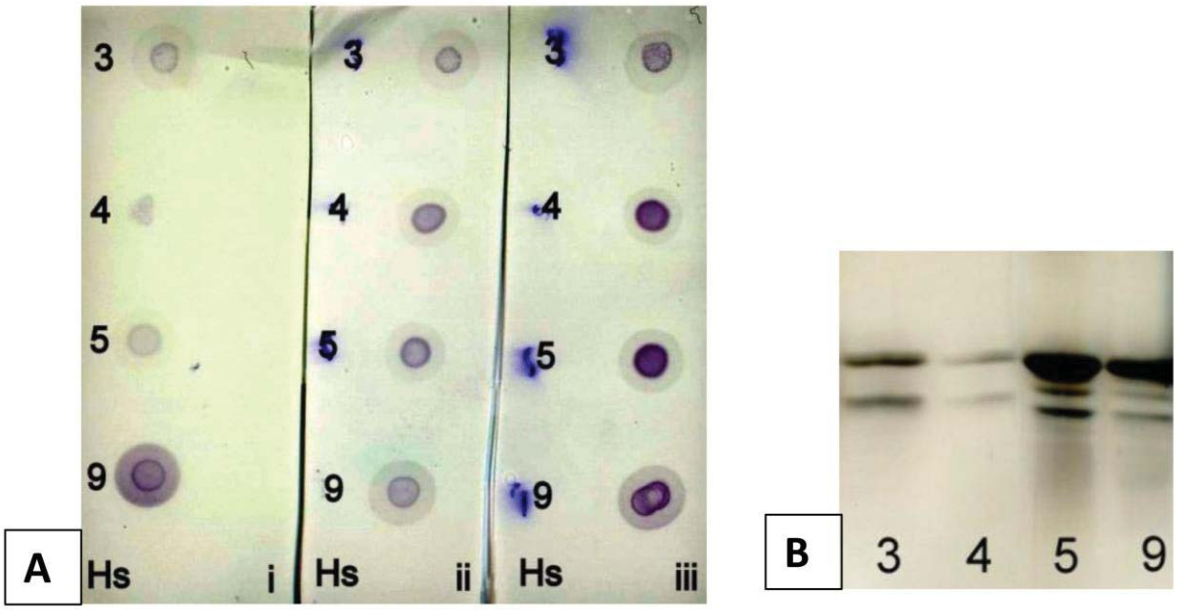


Fig. 7. *H. parasuis* LOS antigenic specificity and electrophoretic profiles. *H. parasuis* serotypes 3, 4, 5, and 9 and *Histophilus* somni (Hs) were grown on agar medium for micro-extraction of the LOS. Each LOS was analyzed by immunobloting (A) with swine antiserum to whole cells of *H. parasuis* serotypes 4 (i), 5(ii), or 9 (iii), and by gel electrophoresis and silver staining (B).

2.4.5 Serum resistance in association with CP expression

H. parasuis serotype 9 grown on agar or in broth to mid-log phase or for 24 h was incubated with buffer only, normal swine serum, precolostral calf serum as a complement source, or calf serum supplemented with antiserum to CP for 60 minutes to assess the susceptibility of

the bacteria to killing by antibody and/or complement (Fig. 8). *H. parasuis* grown on agar were killed by normal swine serum or precolostral calf serum only. However, bacteria grown in broth were resistant to killing by only normal swine serum or precolostral calf serum, but were killed when antiserum to homologous CP was added. Broth-grown *H. parasuis* serotype 9 was not susceptible to killing when antiserum to a heterolgous CP was used. Similar results were obtained with the same serum combinations when serotypes 4 and 5 were tested (data not shown).

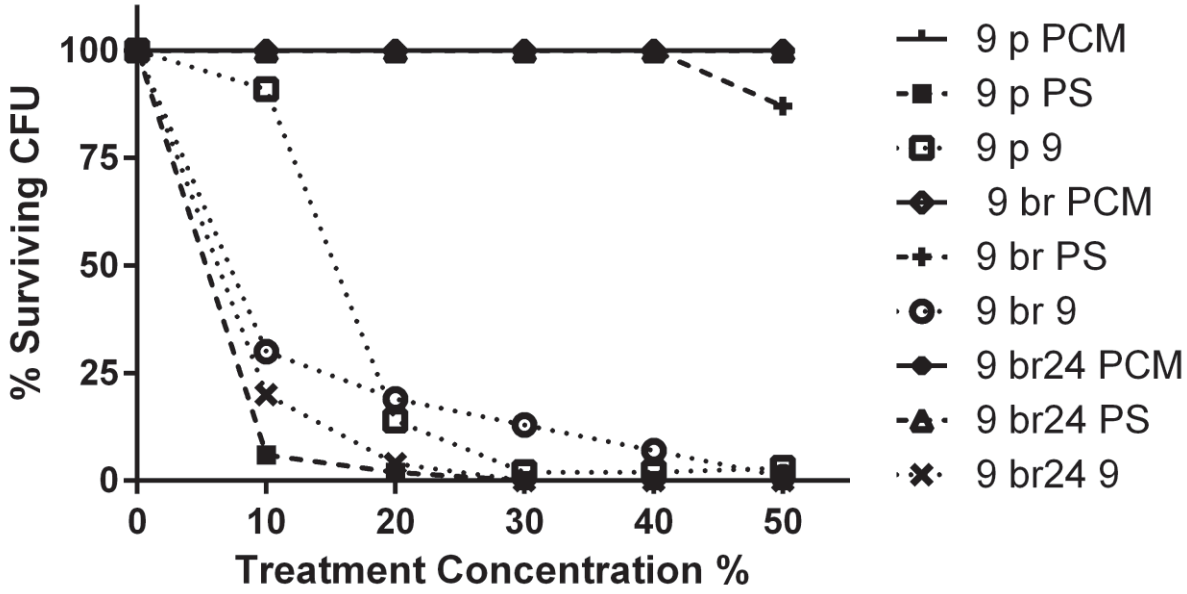


Fig. 8. Bactericidal Assay. *H. parasuis* serotype 9 was grown on agar (p), in broth to mid-log phase (br), or for 24 h (br24). The bacteria were then incubated with buffer (PCM), normal swine serum (PS), or homologous antiserum (9) at increasing concentrations. Pre-colostral calf serum was added as a source of antibody-free complement. The suspensions were mixed and viable plate counts were carried out after 0 and 60 min incubation at 37 °C.

2.4.6 Effect of glucose, FBS, oxygen, or bicarbonate on *H. parasuis* CP expression

H. parasuis serotype 5 was grown in PPLO$^+$ broth without glucose or FBS, or with the addition of Oxyrase. The bacteria were grown to early stationery phase, cell densities normalized, and CP extracted (Fig. 9A). In the absence of glucose supplementation the bacteria failed to grow to early stationery phase (data not shown), but the FBS- and oxygen-deprived cells grew and produced CP in amounts similar to bacteria grown in FBS- and oxygen-rich broth. *H. parasuis* serotype 5 was inoculated to PPLO$^+$ broth supplemented with 3 mM, 10 mM, or 30 mM sodium bicarbonate (Sigma) or with no bicarbonate. The bacteria were grown to early stationery phase, cell densities normalized, and the CP extracted for quantification (Fig. 9B). Bacteria that were grown with 10 mM or more bicarbonate produced significantly more CP than bacteria without bicarbonate supplementation ($p<0.05$ with 10mM bicarbonate, $p<0.01$ with. 30 mM bicarbonate).

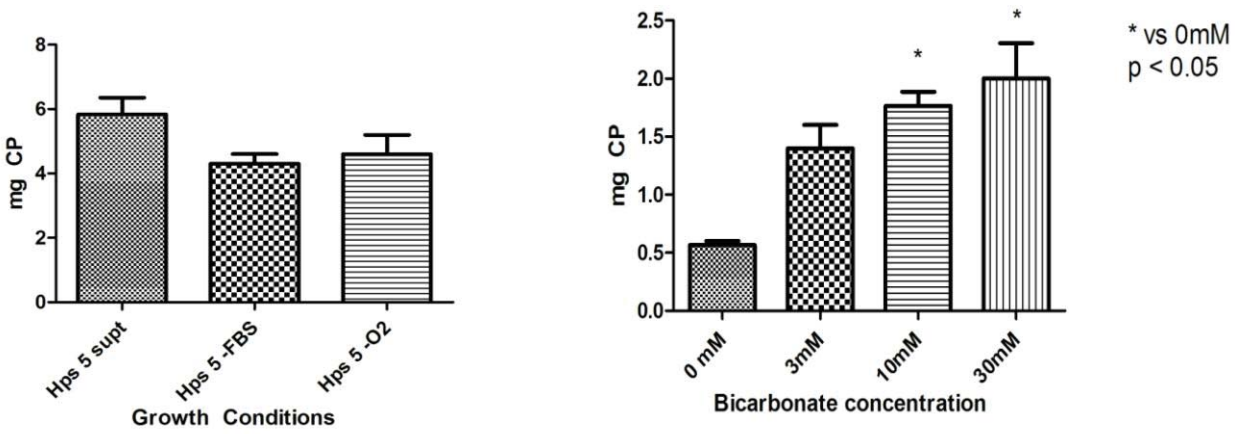


Fig. 9. Growth supplements affecting CP production. (A) *H. parasuis* (Hps) was grown in PPLO$^+$ broth containing 5% fetal bovine serum (FBS), NAD, and 1% glucose (supt); PPLO+ medium lacking FBS (-FBS); PPLO$^+$ medium supplemented with 10% Oxyrase to remove oxygen (-O2); or (B) PPLO$^+$ supplemented with 3-30 mM sodium bicarbonate. * indicates

significantly more CP produced ($p< 0.05$ for 10 mM bicarbonate supplementation, $p<0.01$ for 30 mM bicarbonate supplementation) than the 0 mM bicarbonate control.

When *H. parasuis* serotype 5 was grown on $PPLO^+$ agar or $PPLO^+$ agar to which 30 mM bicarbonate was added, and the bacteria immuno-blotted with antiserum to *H. parasuis* serotype 5 CP (Fig. 10), the bacteria grown on $PPLO^+$ agar supplemented with bicarbonate strongly reacted with antiserum to CP, whereas the bacteria grown on $PPLO^+$ without bicarbonate did not.

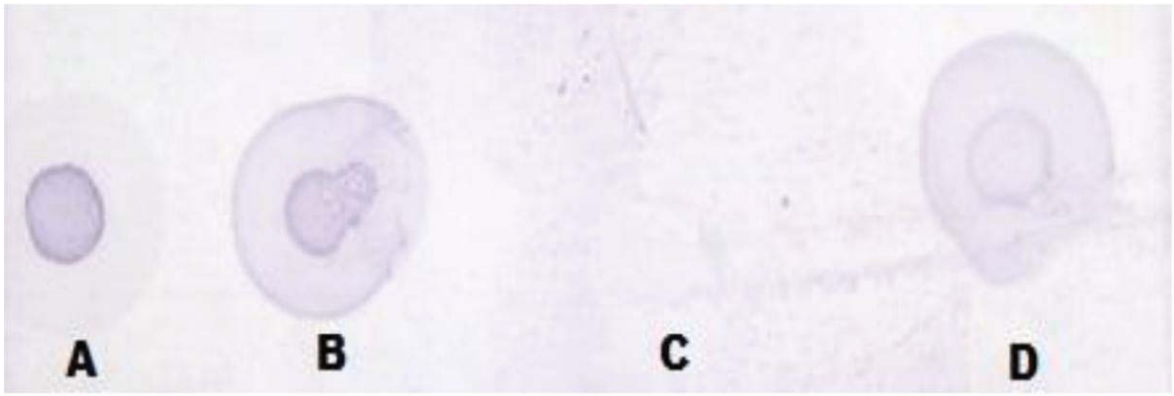


Figure 10. Immuno-blot of *H. parasuis* serotype 5 grown on agar with or without bicarbonate supplementation. A, purified serotype 5 CP; B, broth-grown serotype 5 cells; C, agar-grown serotype 5 cells on $PPLO^+$; D, agar-grown serotype 5 cells on $PPLO^+$ supplemented with 30 mM bicarbonate.

H. parasuis serotype 5 was grown on $PPLO^+$ agar, harvested and used to reculture solid agar medium 11 times before inoculation to $PPLO^+$ broth. The bacteria were grown to early stationery phase and the CP was extracted and compared to CP production by the parent strain (Fig. 11). Even after growth in broth, the bacteria that were passed on agar produced significantly less CP ($p< 0.05$) than the parent strain from both broth culture supernatant and from whole cells.

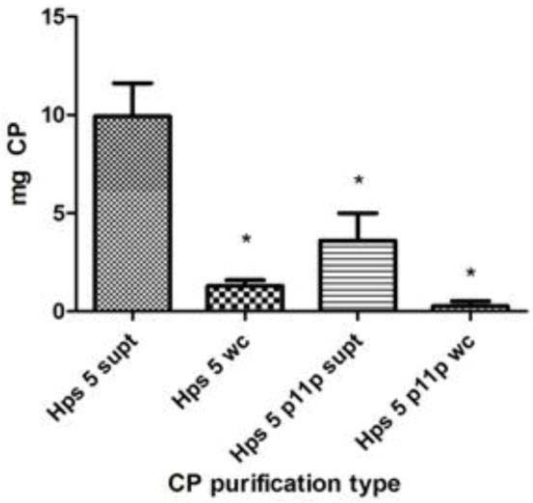


Figure 11. Diminished CP production in broth following repeated passage of *H. parasuis* on agar medium. *H. parasuis* serotype 5 was passed daily on PPLO^{+} agar 11 times, then grown in 250 ml of PPLO^{+} broth, and the CP extracted. Supt, CP isolated from broth supernatant of the non-passed serotype 5 strain; wc, CP isolated from whole bacteria of the parent grown in broth; p11p supt, CP isolated from the broth supernatant of bacteria grown in broth after passage 11 times on PPLO^{+} agar; p11p wc, CP isolated from bacteria grown in broth after passage 11 times on PPLO^{+} agar.

H. parasuis serotype 5 was grown on solid agar medium, suspended in PBS, heated to 121 °C in an autoclave for 90 min to lyse the cells, the lysate was sedimented, and the supernatant and lysate analyzed for CP production in comparison to the equivalent weight of broth-grown cells by ELISA (Fig. 12). The lysed supernatant from agar-grown cells contained CP, but was significantly less than the amount of CP from whole cells that were broth-grown ($p<0.05$). However, the amount of CP extracted from lysed cells was significantly greater than the quantity of CP from unlysed agar-grown cells ($p<0.01$).

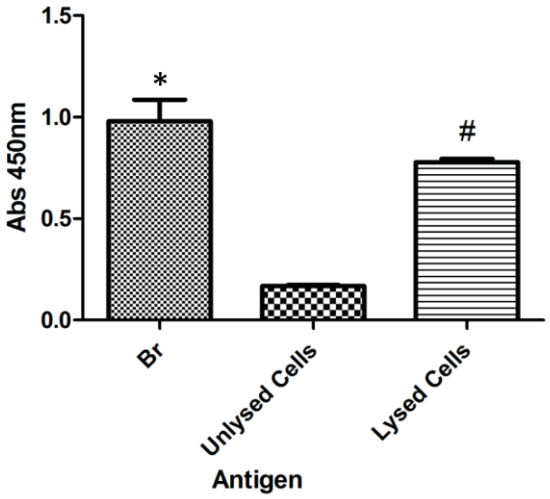


Fig. 12: Production of CP by agar-grown cells. The amount of CP from *H. parasuis* grown in broth (Br) was compared by ELISA to the material extracted from bacteria grown on agar that were lysed or left intact. There was significantly more CP produced by broth-grown bacteria than unlysed agar-grown bacteria (*, $p < 0.05$) and significantly more CP produced by lysed, agar-grown bacteria than unlysed agar-grown bacteria (#, $p < 0.05$). There was no significant difference between CP production by broth-grown bacteria or by agar-grown bacteria ($p > 0.05$).

2.4.7 qRT-PCR for mRNA expression of the CP locus

H. parasuis serotype 5 was grown in $PPLO^+$ broth with shaking for 24 h, and RNA was extracted at 0, 2, 4, 6, 8, 10, 12, and 24 h for qRT-PCR (Fig. 13); *gapA* was used as a reference gene. The Cts of the genes examined at each time-point were compared to those at 0 h to determine relative expression. While many genes in the CP locus were significantly upregulated early in culture, *wza5* was expressed significantly more (4 to 12-fold) when cells were cultured in broth ($p<0.05$) and expression of *iscR5* was significantly greater (> 10-fold) from 12 to 24 h post-inoculation ($p<0.01$) (other time-points not shown).

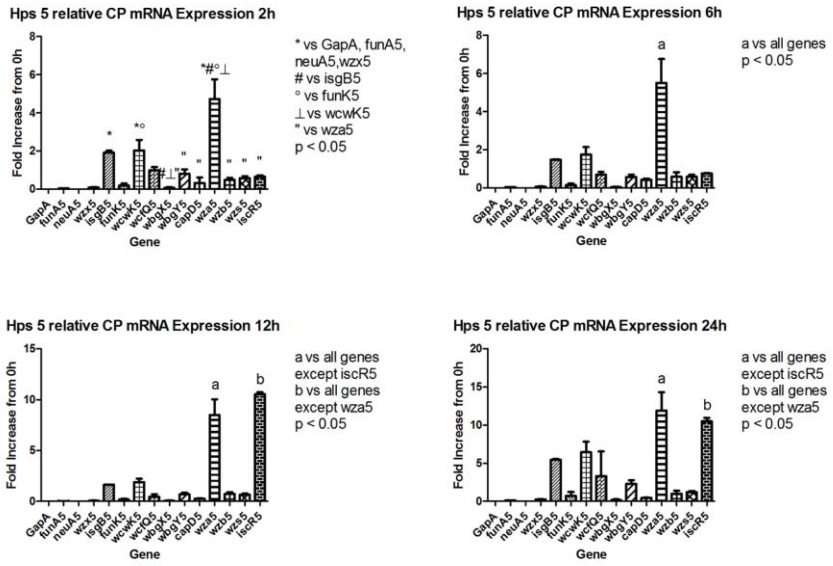


Fig. 13. Relative mRNA expression of *H. parasuis* CP locus genes during growth in broth medium. *H. parasuis* serotype 5 was grown in PPLO^{+} broth, and RNA was extracted from the bacteria after 0, 2, 4, 6, 8, 10, 12, and 24 h growth in broth. The RNA was used for qRT-PCR analysis to measure mRNA expression of the genes in the CP locus. Data at timepoints 4 h, 8 h, and 10 h are not shown.

H. parasuis serotype 5 was grown on PPLO^{+} agar or in broth with shaking for 12 h, both supplemented with 30 mM bicarbonate or with no bicarbonate, and RNA was extracted from the bacteria at 0, 2, and 12 h for qRT-PCR (Table 1) as described above. Many of the CP genes were significantly upregulated by 12 h after addition of bicarbonate to the growth medium, compared to 0 h with no bicarbonate supplementation ($p<0.05$). Of particular note was that *iscR* was more than 10-fold increased in expression by 12 h after the adition of bicarbonate.

Table 1. Expression of CP locus genes by *H. parasuis* serotype 5 with or without bicarbonate supplementation.

	Growth on agar 24 h (fold increase)	Growth in broth 2 h (fold increase)		Growth in broth 12 h (fold increase)	
Gene	**30mM bicarbonate**	**0mM bicarbonate**	**30mM bicarbonate**	**0mM bicarbonate**	**30mM bicarbonate**
funA	8.1658*	0.8429***	0.6876***	0.0325	0.0175
neuA	7.3368*	1.042***	0.9306***	0.0	0.8380[a]
wzx	4.6235*	0.8196***	0.5019**	0.0660	1.459
isgB	4.5814*	0.4855**	0.5349**	1.628*	2.523***
funk	4.5848*	0.9860***	0.4809**	0.1535	4.813***
wcwK	1.3553*	0.1150	0.1188	1.888*	0.2409
wcfQ	5.1110*	0.6219***	1.326**	0.4490	2.595***
wbgX	6.5866*	0.5266***	1.085***	0.0485	3.923***
wbgY	5.4945*	0.4483**	0.9707**	0.6845	0.9909
capD	5.0425*	0.7747***	1.229***	0.2475	0.1059
wza	5.4792*	4.570***	7.457***	8.480***	2.812***
wzb	6.8592*	1.423***	2.218***	0.7185	3.187***
wzs	4.2685*	0.7507**	1.254***	0.5655	1.666*
iscR	3.2512*	0.4411**	1.529***	10.54***	12.39***[a]

H. parasuis serotype 5 strain Nagasaki was grown in $PPLO^{+}$ broth with or without 30 mM bicarbonate supplementation, and RNA was extracted from the bacteria at 24 h growth on agar medium and 2 h and 12 h after growth in broth with bicarbonate supplementation for relative mRNA expression. Asterisks indicate a significant difference between the level of mRNA expression by bacteria grown in broth or in unsupplemented agar medium. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. [a] significant difference in expression between 0 mM and 30 mM bicarbonate.

2.5 Discussion

H. parasuis is an encapsulated, Gram-negative coccobacillus in the family Pasteurellaceae [1]. Fifteen serotypes of *H. parasuis* have been described, as well as untypable strains [9]. The serotype-specific antigen of *H. parasuis* was first isolated by Williamson and

Zamenhof [31], and proposed to be a phosphorylated polysaccharide consisting of *N*-acetylglucosamine and possibly galactose. However, the serotype or strain of *H. parasuis* used was not described. Morozumi and Nicolet [13] also suggested that the type-specific antigen is carbohydrate based on its resistance to heat, pronase, and phenol. Therefore, the leading candidates for the serotype-specific antigen would be LOS or CP. The CP from serotypes 5 and 15 have been isolated and their structures determined by Perry *et al*. [32]. The sugar compositions of these CPs are similar; both contain glucose and neuraminic acid, but differ in that the serotype 5 CP contains galactose-1-phosphate while the serotype 15 CP contains *N*-acetyl-galactosamine-1-phosphate. We also found that the composition of the serotypes 4, 9, and 13 CPs (as well as serotype 5, which was not shown) were similar in composition, but not identical and highly variable in the quantity of individual sugars, suggesting their structures would vary enough from each other to be antigenically distinct. In contrast, the structures of the carbohydrate regions of the LOSs of serotypes 5 and 15 are identical [32].

We have shown that the CP electrophoretic profiles of several *H. parasuis* serotypes were similar to each other and to the well characterized CP of *A. pleuropneumoniae* serotype 5: all were heterogeneous and of large molecular size. Antiserum to each purified CP was serotype specific by immunoblotting, immunofluorescence, immuno-TEM, and ELISA. However, due to the sensitivity of the ELISA, some cross-reactivity between CP serotypes was noted when more concentrated serum was used. This was not unexpected given that the sugar compositions were similar between serotypes. Nonetheless, serotype specificity by ELISA was still obvious when antiserum was adequately diluted. In contrast, LOS from each serotype cross-reacted relatively equally with antiserum to each serotype tested. The limited degree of cross-reactivity that can occur between CPs, the homogeneity between LOS antigens, and the use of antiserum to whole

cells is likely why serotyping of *H. parasuis* can be challenging, with many strains being classified as 2 or more serotypes or untypable [33]. We propose that serotyping should be carried out with antiserum to purified CP to eliminate potentially cross-reactive antigens, or at least with antiserum to a Cetavlon precipitate of cell-free culture supernatant from broth-grown bacteria.

The genes responsible for CP synthesis and export of all 15 serotypes have been described by Howell *et al*. [21]. Of interest, is that the genes in the CP locus are more similar to those in the locus of the group 1 CP from *Escherichia coli* than the group 2/3 CPs of related bacteria, such as *Haemophilus influenza* [34]*, Pasteurella multocida* [35]*, Mannheimia haemolytica* [36]*,* and *A. pleuropneumoniae* [37]. For example, all serotypes of *H. parasuis* contain *wza, wzb*, and *wzs* (homologue of *wzc*)*,* and most contain *wzx* in their CP loci [21]. The proteins Wzb, Wzc, and Wza are proposed to be involved in phosphorylation and transport of the CP across the inner and outer membranes to the bacterial surface. Wzx is required to flip the undecaprenol pyrophosphate-linked polymers across the inner membrane [38]. In contrast, group 2/3 CPs are exported by an ATP-binding cassette transport mechanism [39]. These results, among others, bring into question the current taxonomic classification of *H. parasuis*, which is under further investigation.

Wang *et al*. [40] mutated the *capD* gene in *H. parasuis* strain SHO165, serotype 5, and showed that the *capD* deletion mutant was attenuated in pigs and highly sensitive to complement-mediated serum killing. Although the authors did not report if there was any change in CP expression by the *capD* mutant, *capD* has been identified as part of the CP biosynthesis region in 13 of the 15 serotypes examined (serotypes 3 and 4 are exceptions) [21]. We obtained similar bactericidal results when serotypes 4, 5, and 9 were grown on agar medium and CP was not expressed; all were susceptible to killing by serum complement alone. However, as reported

for *H. influenzae* type b [11], when antibody specific to the homologous capsule was added, broth-grown bacteria expressing CP were efficiently killed, whereas the bacteria were serum-resistant in the absence of specific antibody.

Although the amount of CP expressed by bacteria can be enhanced by environmental factors, such as aeration and glucose supplementation [41, 42], CPs are normally constitutively expressed. Therefore, we were surprised to find that no CP could be isolated from any of the *H. parasuis* serotypes grown on agar medium, but CP was expressed on the surface of the bacteria and shed into the culture supernatant when grown in broth medium. Furthermore, the lack of CP surface expression appeared to occur at the level of CP transport rather than synthesis, based primarily on the detection of CP in lysed bacteria grown on agar medium. Preparation of agar-grown *H. parasuis* for serotyping by autoclaving is commonly used (J. Gallant, personal communication), and will lyse the cells thereby releasing the type-specific CP. Many *H. parasuis* strains are described as untypable, including some same strains that are typed by one laboratory and reported as untypable by another laboratory (V. Aragon, personal communication). Therefore, how the laboratory prepares the bacteria for serotyping will affect whether CP can be detected or not. The simplest, most efficient procedure would be to grow the bacteria in a suitable broth medium and use the entire culture, or the cell-free supernatant only, for typing.

Further evidence that CP expression is inhibited at the level of transport was provided by qRT-PCR. Several genes in the CP locus were upregulated over a 24 h growth period in broth. However, within 2 h of growth in broth medium the export gene *wza* was upregulated 4-fold and continued to be upregulated more than other genes in the locus over the next 24 h. After the bacteria were grown for 12 h in broth *iscR*, which is immediately downstream of the last export gene *wzs*, was upregulated more than *wza* (>10-fold) and continued to be upregulated at that

level through 24 h growth in broth. Furthermore, in the presence of bicarbonate *iscR* was further upregulated more than other genes after 12 h growth in broth. In *E. coli* IscR is an iron-sulfur transcription factor that regulates the *isc* operon, and affects the expression of a large number of genes responsible for diverse metabolic and respiratory functions, and biofilm formation [43]. Therefore, an attractive hypothesis is that *iscR* is involved in regulating transport of CP in *H. parasuis*. The specific mechanism by which *iscR* and other genes regulate CP transport in *H. parasuis* is currently under investigation.

Aeration, glucose supplementation, or FBS supplementation did not contribute to the "all or none" expression of CP. However, supplementation of the medium with sodium bicarbonate enhanced CP expression in a dose-dependent manner. Of particular interest was that bicarbonate supplementation not only enhanced CP production in broth medium, but supplementation of bicarbonate to the agar medium resulted in expression of CP on agar-grown bacteria as well. Carbonic anhydrase catalyzes the interconversion of bicarbonate and carbon dioxide. *H. parasuis* requires CO_2 for growth on agar medium, but not in broth, presumably due to its own carbonic anhydrase (gene 1919590..1920252/locus tag JL26_09770; strain SHO3). In *Streptococcus pneumoniae* a carbonic anhydrase mutant can grow when the medium is supplemented with bicarbonate, but not in its absence [44]. Furthermore, growth of the *S. pneumoniae* carbonic anhydrase mutant could be partially restored by unsaturated fatty acids, indicating a link between carbonic anhydrase and unsaturated fatty acid synthesis. CPs are thought to be anchored to the cell wall through a terminal membrane lipid [45]. Therefore, enhanced lipid synthesis may promote CP expression on the surface of *H. parasuis*, though it is not clear at this time how lipid biosynthesis would be related to CP export in *H. parasuis*.

Bicarbonate is also released from mammalian cells during times of physiological stress to manage host pH [46]. Stressful conditions that exist for piglets and adult pigs include weaning, transport, and previous infections. Bicarbonate may act as a reporter ligand for the bacteria and trigger changes in their metabolism in response to signal changes in the host environment. Bicarbonate interacts with trans-membrane adenylate cyclase cell receptors, resulting in activation of kinase cascades within the cell and potentially creating downstream phosphorylated products that can regulate gene expression, including genes responsible for CP synthesis and transport [47]. Therefore, the presence of bicarbonate in systemic sites of the host could enhance the amount of CP produced and expressed on the bacteria, thereby enhancing virulence by protecting the bacteria from host defenses.

In addition to irregularities in serotyping, some strains/serotypes of *H. parasuis* are regarded as less virulent or avirulent. However, challenge of pigs with an "avirulent" serotype occasionally results in disease [48, 49]. Again, variable expression of CP could explain this discrepancy because the absence of CP on the bacteria would make them unable to disseminate. *H. parasuis* likely colonizes various sites of the upper respiratory tract of young pigs [1]. In the upper respiratory track they may express colonizing adherence factors that may be obscured if CP is expressed. However, in order to disseminate and cause multi-tissue infection, expression of CP would be required to protect *H. parasuis* in the bloodstream and tissues by evading opsonization and bactericidal factors. The presence of sialic acid on the CP would further contribute to evading detection by the immune system, as sialic acid is a normal component of host tissues, and is non-immunogenic [50]. It is likely that growth in broth may more closely mimic physiological systemic conditions during dissemination [51]. Further molecular analyses are being carried out to determine the regulatory mechanisms responsible for CP production and

transport. Investigation of the regulation of CP will aid in understanding how control over expression of CP promotes survival of *H. parasuis* in its only known natural host.

2.6 Acknowledgements

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Chapter 3

A Latex Agglutination Diagnostic for Rapid Detection and Serotyping of *Haemophilus parasuis*

Anne C. Michalenka Hyman[1]
Thomas J. Inzana[1,2]

[1] Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA.
[2]Virginia Tech Carilion School of Medicine, Roanoke, Virginia, United States of America

3.1 Abstract

Haemophilus parasuis is the etiologic agent of Glässer's Disease in piglets and pneumonia in adult pigs from clean herds. This bacterium is responsible for infection that leads to economic loss for swine producers. *H. parasuis* infection causes a broad spectrum of pathologies and the disease is often fatal, and difficult to treat empirically without proper diagnosis. Current diagnostic measures for determining *H. parasuis* infection require growth on agar medium, but may take days to complete, risking decimation of a compromised herd. A more rapid, efficient, and reliable diagnostic method is needed to bolster the health of the swine herds suseptible to *H. parasuis* infection. We have recently shown that the capsular polysaccharide (CP) of *H. parasuis* confers serotype specificity, and have previously demonstrated that IgG antibodies to specific CPs of other bacteria can be coupled to latex beads to produce an agglutination response when mixed with positive samples. We propose that a similar latex agglutination test using IgG antibodies against *H. parasuis* CPs conjugated to latex beads will yield rapid and specific results for diagnostic and serotyping purposes. IgG antibodies from rabbit serum made against several purified *H. parasuis* CPs were coupled to latex beads (SLPs), and the SLPs were assayed for IgG retention. Cells and purified CPs from several reference *H. parasuis* serotype strains were used to test the efficacy of the SLP assay, by directly mixing a suspension of *H. parasuis* material with the SLPs and observing the amount of agglutination within a particular timeframe. Cells grown on agar were lysed by either autoclaving or boiling, and their lysates were used to determine the limit of detection for these cell preparations. Clinical *H. parasuis* isolates acquired from a diagnostic lab were typed using SLPs, and each serotype was confirmed by PCR using primers to serotype-specific genes. Negative controls from bacteria involved in swine diseases were also mixed with the SLPs to determine the sensitivity of the

assay. IgG retention on the SLPs was over 90% for each serotype. The limit for homologous detection was between 15 and 150 pg of purified CP, depending on the serotype, and 1.5 x10^5 CFU of cells. A weaker agglutination response was observed with heterologous serotype CPs or cells of higher concentrations, but no agglutination reactivity with non-*H. parasuis* bacteria was observed at any concentration. Agar-grown cells that were autoclaved reacted with the SLPs, and the boiled cells also induced a slightly attenuated agglutination response compared to the autoclaved cells, whereas unlysed agar-grown cells did not cause any agglutination. The average threshold for specific serotype recognition by homologous SLPs is 1.5 x 10^6 CFU. The sensitivity of identification of 30 clinical isolates with SLPs specific to serotypes 4, 5, and 9 is 90.1%, even though some of the isolates were initially atypeable due to mixed cultures of *H. parasuis* strains or strains with Gram-positive contamination. The specificity of the SLP assay with 27 non-*H. parasuis* swine disease-related bacteria with SLPs specific to serotypes 4, 5, and 9 is currently 100%. The latex agglutination assay demonstrates a solution to the problem of slow and inconsistent diagnostic and typing methods that are currently available, and will help direct future research in disease prevention and serotype tracking in *H. parasuis* infections.

3.2 Introduction

Haemophilus parasuis is the etiologic agent of Glässer's Disease in piglets and pneumonia in adult pigs from clean or specific pathogen-free herds. This bacterium is responsible for infection that leads to widespread economic devastation for swine producers worldwide. Current methods of prevention include vaccination, but the vaccine does not protect against all known virulent serotypes [1]. *H parasuis* infection causes a broad spectrum of pathologies, including polyserositis, meningitis, pericarditis, pleuritis, and sepsis, and the disease

can often be fatal [2]. Glässer's Disease is treatable with a course of β-lactam antibiotics, but because of the broad spectrum of presenting pathologies, *H. parasuis* infection is difficult to treat empirically without proper diagnosis. Current diagnostic measures for confirming *H. parasuis* infection in pigs require growth on agar medium and confirmation by PCR [3], but because of the fastidious nature of *H. parasuis* this diagnostic procedure may take days to complete. Most swine infected with *H. parasuis* succumb to disease within 72 hours, and the proper diagnosis for a compromised herd may come too late to prevent most of the herd from decimation due to infection [4]. A rapid field diagnostic test would greatly improve the protection and overall health of swine herds that are susceptible to *H. parasuis* infection.

To date, fifteen serotypes of *H. parasuis* have been characterized by immunodiffusion [5]. Although these fifteen serotypes have defined characteristics and are most are typable, many clinical isolates are unable to be serotyped by currently available methods [6]. Moreover, the number of false positive serotype designations also gives rise for concern to the validity of current typing methods. A more consistent method of serotyping would be valuable for tracking the prevalence of virulent serotypes and strains within serotypes for epidemiologic information. Even though the immunodiffusion method used for *H. parasuis* serotype designation is currently still used in serotyping isolates [2] , the antisera used in this assay were made against whole cells of *H. parasuis*. A monospecific antiserum could be more effective at accurately serotyping clinical isolates. The antigenic determinant for the fifteen serotypes had not been characterized until recently [7]. The capsular polysaccharide (CP) of *H. parasuis* is the serotype specific antigen, and antisera to CP reacts significantly with its homologous CP found on cells grown in broth culture or in cells grown on solid agar medium.

We have shown previously that purified IgG antibodies specific to the CP of *Actinobacillus pleuropneumoniae* can be conjugated to latex particles to produce a rapid agglutination response when mixed with *A. pleuropneumoniae*-specific samples [8]. We propose that a similar latex agglutination test utilizing IgG antibodies conjugated to latex particles will yield rapid and specific results for diagnostic and serotyping purposes for *H. parasuis*, and will have a valuable role in swine health, bacterial taxonomy and epidemiology.

3.3 Materials and Methods

3.3.1 Bacteria Strains

Reference strains of *H. parasuis* serotypes 4, 5, and 9 were acquired from Dr. Linda Zeller from the Iowa State School of Veterinary in Ames, IA. Clinical isolates of *H. parasuis* were obtained from Dr. Karen Post at Rollins Diagnostic Laboratory in Raleigh, North Carolina. Isolates from swine diagnosed with infections due to non-*H. parasuis* pathogens were also obtained from Rollins Diagnostic Laboratory, as well as the Clinical Diagnostic Laboratory at the Virginia-Maryland College of Veterinary Medicine. (Table 1). *A. pleuropneumoniae* serotype 5 strain J45 was used as a negative control [8].

Table 1. Isolates used for latex agglutination assay.

***H. parasuis* isolate (and tissue source if available)**	**Non-*H. parasuis* isolates**
SW124, serotype 4 (nose)	*P. multocida 11378*
Nagasaki, serotype 5 (meninges and sera)	*P. multocida OSU*
D74, serotype 9	*P. multocida* 96020298
5013-96	48724 BO
41363	49016 A1
W94-6357	*Corynebacterium* spp.
W94-9737	*A. pleuropneumoniae* serotype 1
15945-96 (lung)	*A. pleuropneumoniae* serotype 2
46072	*A. pleuropneumoniae* serotype 3
161151-96	*P. aeruginosa*
4884-96	545-100

W94-45875	7453-3
W95-46072 S-9	*E. coli*
22309	*C. perfringens*
10194-96	*S. typhimurium* 5096
W94-45548	*S. typhimurium* 5172
W94-47951	*S. typhiurium* 2
7033-96	*P. suis*
11524-96	*E. faecalis*
45510	*S. dysgalactiae*
6249-96	*S. aureus*
3034-96	*A. pleuropneumoniae* J45
3548-96	
12783-96	
11166-96 (lung)	
6901-96	
W94-46462	
W94-46463	
W95-572	

3.3.2 Bacterial growth

H. parasuis cells were grown on solid PPLO agar medium supplemented with 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 1% glucose (Sigma, St. Louis, MO) and 100 µg/mL nicotinamide adenine dinucleotide (Sigma) ($PPLO^+$), for about 48 h at 37°C and were either scraped into phosphate buffered saline supplemented with 0.15 mM calcium chloride and 0.09 mM magnesium chloride (PCM, Sigma) or used to inoculate $PPLO^+$ broth. Agar-grown cells used directly in agglutination assays were either left intact or lysed by autoclaving at 121°C for 90 min or boiling for 10 min, and cell lysates were separated from the supernatant by centrifugation at 8,000 x *g* for 15 min at 4°C. Cells in broth medium were grown in 20 mL $PPLO^+$, rotating in flasks at 200 rpm at 37°C for 4 h until mid-log phase density of 10^9 CFU/mL, determined spectrophotometrically, or 24 h until the end of growth phase. Cells were separated from broth medium by centrifugation at 8,000 x *g* for 15 min, at 4°C. The supernatants were

saved and cells were resuspended in PCM for storage until agglutination analysis. *A. pleuropneumoniae* serotype 5 cells were cultured in the same manner, as a negative control.

3.3.3. CP purification

Briefly, CPs were isolated from *H. parasuis* serotypes 4, 5, and 9 using Cetavlon precipitation, enzyme digestion, and phenol extraction as previously described [7]. Samples were sedimented by ultracentrifugation at 41,000 x *g* to remove any remaining LOS, and the supernatant was lyophilized. CPs were stored in PCM at a concentration of 10 mg/mL. CP from *A. pleuropneumoniae* strain J45 was isolated as previously described [9].

3.3.4 CP antisera generation and Immunoglobulin G purification

Antisera were generated against CPs of *H. parasuis* serotypes 4, 5, and 9 as previously described [7], and all animal work was completed humanely and following the standards recommended by the Institutional Animal Care and Use Committee under protocol 12-073-CVM. Briefly, three rabbits were immunized subcutaneously with 100 μg of purified CP from serotype 4, 5, or 9 in sterile PBS mixed 1:1 with Freund's Complete adjuvant (Sigma). Blood samples were obtained prior to immunization to establish baseline antibody titers to CP, and then subsequently every two weeks to monitor antibody titers. Animals were boosted with the same concentration of the homologous CP in Freund's Incomplete adjuvant 4 weeks post-inoculation, then intravenously in sterile PBS 4 weeks thereafter, and then every 2 weeks until the antibody titer was over 1:10,000, as determined by enzyme-linked immunosorbent assay (ELISA).

Immunoglobulin G (IgG) purification was performed using hyperimmune rabbit serum against *H. parasuis* serotypes 4, 5, or 9. The sera were individually passed through a Protein A/G

affinity purification column (AmiconCorp., Danvers, MA) to isolate the IgG component. The IgG concentration was determined by Bicinchoninic Acid Assay (BCA, Pierce, Fairlawn, NJ) following the manufacturer's protocol. The anti-CP IgG concentration of each serotype was greater than 1.4 mg/ml.

3.3.5 Coupling of IgG to carboxylated latex beads

Affinity-purified antibody to CP was coupled to carboxylate latex particles 0.75 mm in diameter (Polysciences,Inc., Warrington, PA) by modification of procedures recommended by the manufacturer. The latex particles (0.5 ml of solution) were washed three times in 0.1 M carbonate buffer (pH = 9.6), followed by three washes in 0.02 M phosphate buffer (pH = 4.7). All washing steps were completed in 1.5 ml microcentrifuge tubes by resuspension of material with a sterile pipet tip, followed by centrifugation at 14,000 x *g* for 6 min at room temperature unless otherwise stated. Following the final wash, the particles were resuspended in 0.625 ml of the phosphate buffer and transferred to 1.8-ml cryovials (Nalgene, Rochester, NY), and 0.625 ml of a freshly prepared solution of 2% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC; Bio-Rad Laboratories, Richmond, CA) was added dropwise while the solution was slowly vortexed. The tubes were rotated slowly end-over-end for 4 h at room temperature, and the SLPs were transferred to microcentrifuge tubes and washed three times with 0.01 M borate buffer (pH = 8.0). The SLPs were resuspended in 1.2 ml of borate buffer and transferred to clean 1.8 ml cryovials. IgG antibodies at a concentration of 1 mg/0.5 ml of particles were added to the SLPs, and were then rotated end-over-end overnight at room temperature. To block nonspecific binding, 50 µl of 0.25 M ethanolamine was added to the SLP-IgG reaction and rotation was continued for 30 min. The SLPs were transferred to 1.5 ml microcentrifuge tubes and centrifuged

for 10 min at 14,000 x *g*. The supernatant was saved for protein determination by BCA assay. Following conjugation of IgG antibodies to the SLPs, it was determined that over 90% of anti-CP IgG to each serotype was bound to their respective SLPs. The SLPs were resuspended in 1% bovine serum albumin in borate buffer and rotated for 30 min at room temperature. The SLPs were washed one additional time in BSA and resuspended in 0.5 ml of latex storage buffer (1% [wt/vol] BSA, 5% [vol/vol] glycerol, and 0.1% (wt/vol] NaN_3 in PBS).

3.3.6 Latex Agglutination Assay

CP resuspended in PCM, bacteria resuspended at a concentration of 10^9 CFU/ml in PCM, and bacterial cell lysates were used in the SLP agglutination assays. Each sample of material was diluted 1:10 serially in PCM for at least 7 dilutions. The latex agglutination test was performed on a glass slide. Several different volumes of 10 mg/ml concentration CPs and SLPs were used in trial to optimize the efficiency of agglutination within two minutes, until ratio of CP to SLPs was found to agglutinate within 2 minutes of combination. Five µl of SLPs were mixed with 15 µl of a sample of CP or cell preparation. The slide suspensions were mixed manually using a sterile pipet tip and then rotated slowly to ensure homogeneity of the sample. Rotation of the slides occurred for 2 minutes, and then an agglutination score was assigned to each sample. Agglutination scores were dependent on the percentage of observed agglutination: 0- no agglutination, 1- indeterminate agglutination, 2- ≥ 25% agglutination, 3- ≥50% agglutination, 4- ≥ 75% agglutination. Clear agglutination was indicated by a score of 2 or higher. Each agglutination assay was repeated at least three times. The limit of detection for CP or cell preparation was defined as the smallest concentration of CP or cell preparation that achieved a score of 2 or higher during the agglutination assay. Limits of detection for homologous serotypes

was defined as the smallest concentration of CP or cell preparation that achieved a score of 2 or higher during the agglutination assay, while the heterologous SLP agglutination scores were less than 2.

Clnical isolates were mixed with each type of SLP as stated above, and agglutination was observed and assigned a score. If the agglutination score for more than one serotype-specific SLP was higher than 2, the sample was diluted, and the assay was repeated until only one specific SLP had an observed agglutination score of 2 or higher.

3.3.7 PCR Confirmation of serotype specificity

Genes from the capsule loci of *H. parasuis* serotypes 4, 5, and 9 [10] were evaluated for genetic uniqueness using the nucleotide Basic Local Alignment Search Tool (National Center for Biotechnology Information, Bethesda, MD), and primers for each gene with similar melting temperatures were designed using Primer Blast (NCBI). These oligonucleotide sequences were used to create primers for PCR (IDT, Coralville, IA) for serotype confirmation (Table 2). DNA was extracted from *H. parasuis* cells using the DNEasy Blood and Tissue kit (Qiagen, Valencia, CA), following manufacturer's instructions for DNA extraction from Gram-negative bacteria. DNA was mixed with OneTaq DNA polymerase (New England Biolabs, Ipswich, MA), dNTPs (Life Technologies, East Rutherford, NJ), and appropriate reagents following manufacturer's instructions, and PCR was performed on the DNA samples in an Eppendorf Mastercycler Pro thermocycler (Eppendorf, Hauppage, NY), with one initial step cycle at 94 °C for five minutes, followed by 30 cycles of denaturating to 94 °C for 30 sec, annealing at 58 °C for 60 sec, and elongation at 68°C for 60 sec. A final elongation step was performed for 5 min at 68 °C. The PCR products were mixed with DNA loading buffer (New England Biolabs), and were

electrophoresed on a 1% agarose/TAE gel treated with 0.01% ethidium bromide (Fisher Scientific) at 100 V until the samples had run sufficiently for gene product detection. Gene products were viewed and photographed under UV light on a GelDoc XR (BioRad, Hercules, CA).

Table 2: Primers for PCR serotype confirmation.

Gene[a]	Forward primer	Reverse primer	size (bp)
gltG, 4	CAAGTGAATGCTAACCTTGGGT	ACTCCATTGCCATTTAGCTGC	164
wcwK, 5	CTGGATAGAGAGTGGCAGGC	TCCATCAGGGCACCTGGAC	541
gltM, 9	ACACCATCTCCAACACCGAAA	CCCTGGCACGTCAGTAA	796

[a]Genes identified from the CP loci of *H. parasuis* serotypes 4, 5, and 9 that were unique to each specific locus.

3.3.8 Data processing and statistical analyses

All data points were recorded and student's t-test and 2-way ANOVA with Bonferroni post-test calculations were determined using InStat GraphPad Prism v. 5.02 to determine statistical significance. Percent sensitivity was calculated with the formula [# true positives/(true positives + false negatives)] x 100, and % specificity was calculated with the formula [#true negatives/(true negatives + false positives) x 100, Fisher's Exact test to calculate the confidence interval of sensitivity and specificity, scatterplots and histograms were also generated with GraphPad Prism.

3.4 Results

3.4.1 Limit of detection of SLPs with Homologous and Heterologous CPs

Clear agglutination (a score of 2+) occurred with 15 pg of CP from *H. parasuis* serotypes 4 and 5, and 150 pg of CP from serotype 9. *A. pleuropneumoniae* strain J45 CP did not agglutinate any of the SLPs at a concentration of 150 µg (Fig. 1).

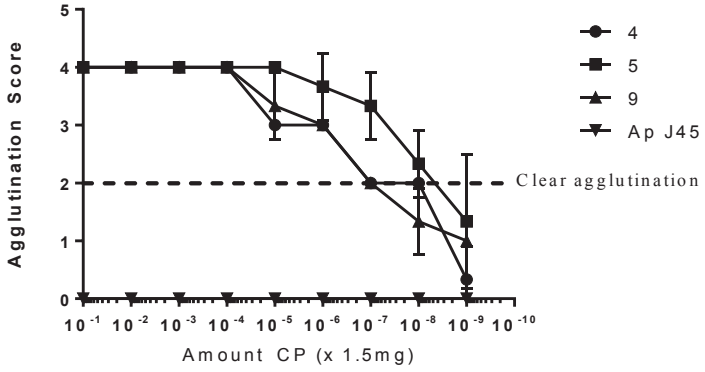

Figure 1: Reactivity of SLPs with homologous CPs. Serial dilutions of purified CPs from *H. parasuis* serotypes 4, 5, and 9 and *A. pleuropneumoniae* strain J45 were mixed with SLPs conjugated to anti-CP IgG of the homologous serotype and agglutination scores were assigned based on % agglutination after 2 minutes. Clear agglutination was defined as a score of 2+, or over 25% agglutination.

Purified CPs from serotypes 4, 5, and 9 were mixed with heterologous SLPs and agglutination was scored after 2 minutes (Fig. 2). For concentrations between 150 µg and 1.5 µg of CP, each serotype-specific SLP agglutinated with other CP serotypes. The CP concentrations were diluted until agglutination scores of 2+ were only observed with the serotype-specific SLP. The thresholds for specific agglutination were 1.5 ng for serotypes 4 and 5, and 1.5 µg for serotype 9.

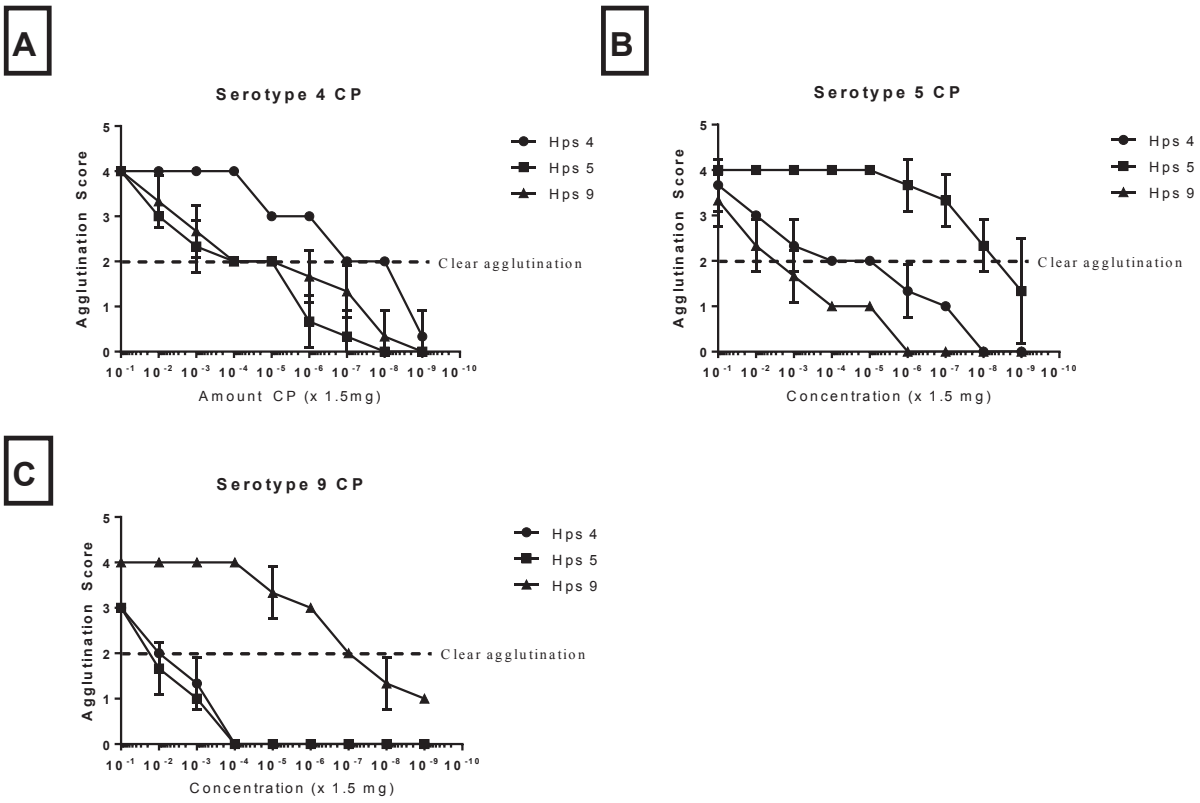

Figure 2: Reactivity of SLPs with heterologous CPs. Serial dilutions of serotypes 4 (A), 5 (B), and 9 (C) were mixed with SLPs conjugated to anti-CP IgG for each listed serotype. Agglutination scores were assigned based on % agglutination after 2 minutes. Clear agglutination was defined as a score of 2+, or over 25% agglutination, and the threshold of serotype-specific detection was defined as the concentration where only the homologous serotype CP exhibited clear agglutination.

3.4.2 Limit of detection of SLPs with homologous and heterologous broth-grown *H. parasuis*

Cells from *H. parasuis* serotypes 4, 5, and 9 and *A. pleuropneumoniae* strain J45 were grown in broth culture, washed, mixed with SLPs, and the mixtures were evaluated for agglutination. Each sample (1.5×10^7 CFU/ml and subsequent dilutions) was mixed with SLPs containing the homologous IgG for 2 min (Fig. 3). The limit of detection of the SLPs with each

homologous serotype was about $1.5x10^5$ CFU. *A. pleuropneumoniae* strain J45 cells at 1.5×10^7 CFU did not agglutinate any of the SLPs.

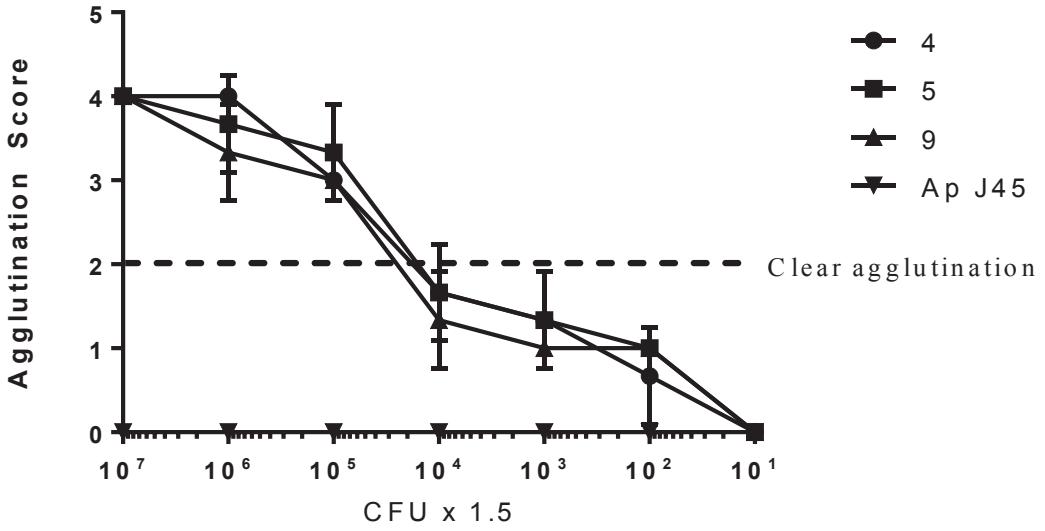

Figure 3. Reactivity of SLPs with homologous cells. Serial dilutions of broth-grown cells from *H. parasuis* serotypes 4, 5, and 9 and *A. pleuropneumoniae* strain J45 were mixed with SLPs conjugated to anti-CP IgG of the homologous serotype and agglutination scores were assigned based on percent agglutination after 2 minutes. Clear agglutination was defined as a score of 2+, or over 25% agglutination.

The same broth-grown *H. parasuis* cells were also mixed with heterologous serotype-specific SLPs and agglutination scores were assigned to each sample (Fig. 4). For concentrations greater than 10^7 CFU, each serotype-specific SLP agglutinated with multiple cell serotypes. The samples concentrations were diluted until agglutination scores of 2+ were only observed with the serotype-specific SLP. The thresholds for serotype-specific agglutination were 1.5×10^5 CFU for serotypes 4 and 5, and 1.5×10^6 for serotype 9.

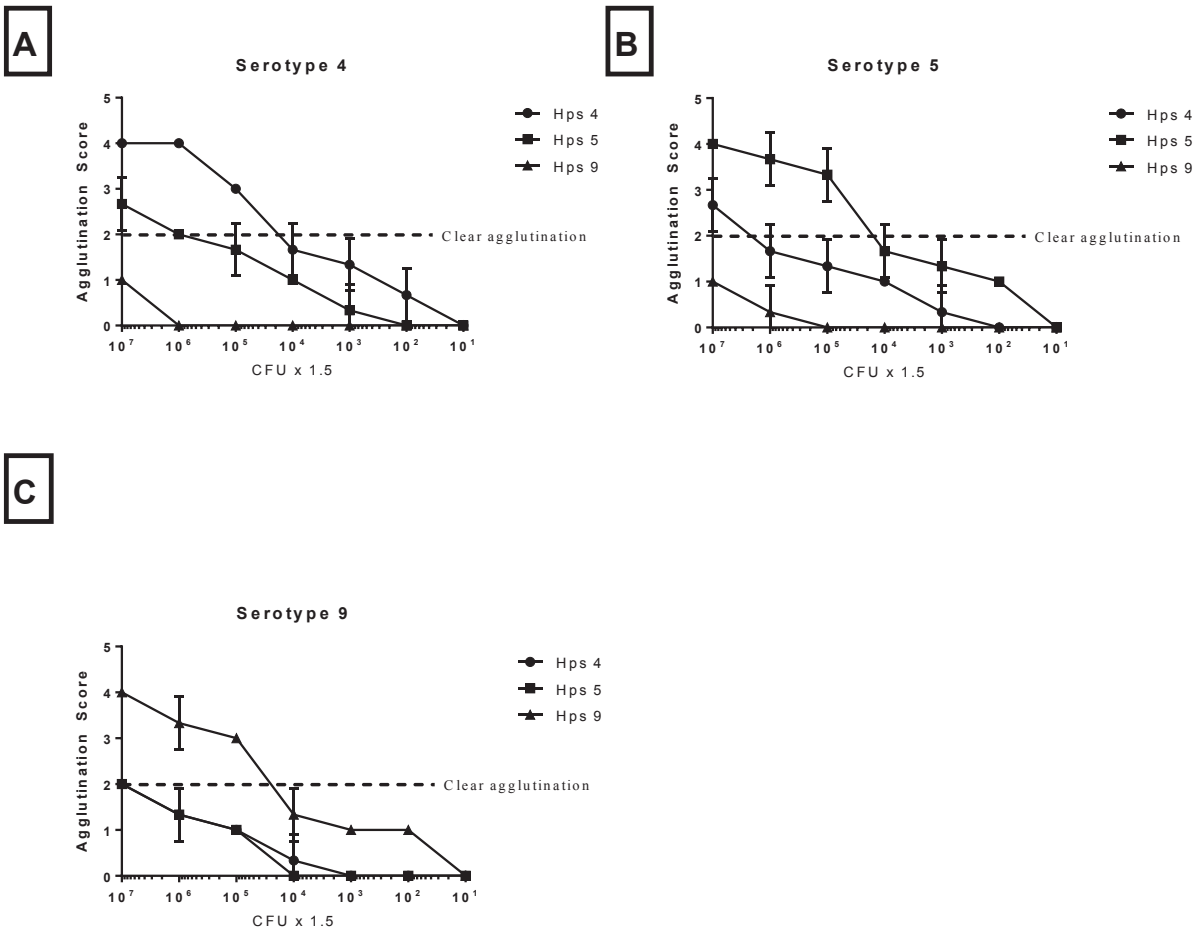

Figure 4. Reactivity of SLPs with heterologous cells. Serial dilutions of cells from *H. parasuis* serotypes 4 (A), 5 (B), and 9 (C) were mixed with SLPs conjugated to anti-CP IgG for each listed serotype. Agglutination scores were assigned based on % agglutination after 2 minutes. Clear agglutination was defined as a score of 2+, or over 25% agglutination, and the threshold of serotype-specific detection was defined as the concentration where only the homologous serotype exhibited clear agglutination.

3.4.3 Effect of growth medium and cell lysis on SLP agglutination.

When the bacteria were scraped from solid agar medium and suspended at 10^9 CFU/ml, no agglutination of SLPs was detected (Fig. 5). However, if the agar-grown bacteria were first

lysed by autoclaving for 90 min or boiling for 10 min, the SLPs did agglutinate. CP from the bacteria autoclaved could be detected at 1.5 x 10^4 CFU/ml, whereas CP from boiled bacteria could only be detected at 1.5 x 10^5 CFU/ml (Fig. 6).

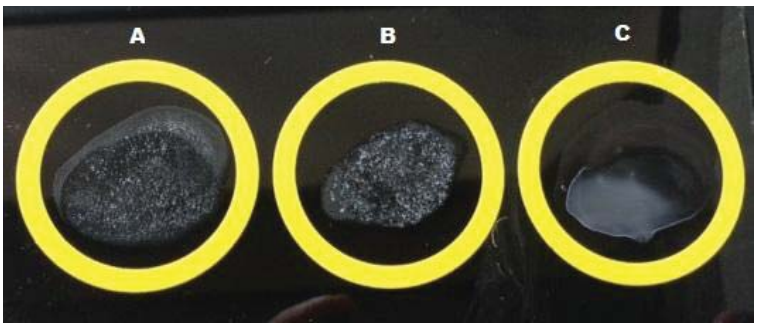

Figure 5: Agglutination of *H. parasuis* serotype 5 whole cells. Five µl of latex particles to serotype 5 were mixed with 150 µg of purified serotype 5 CP in 15 µl (A), 1.5 x 10^7 CFU of broth-grown cells or agar-grown lysed by autoclaving or boiling for 10 min (B, representative of all lysates), or 1.5 x 10^7 CFU of agar-grown, unlysed cells (C).

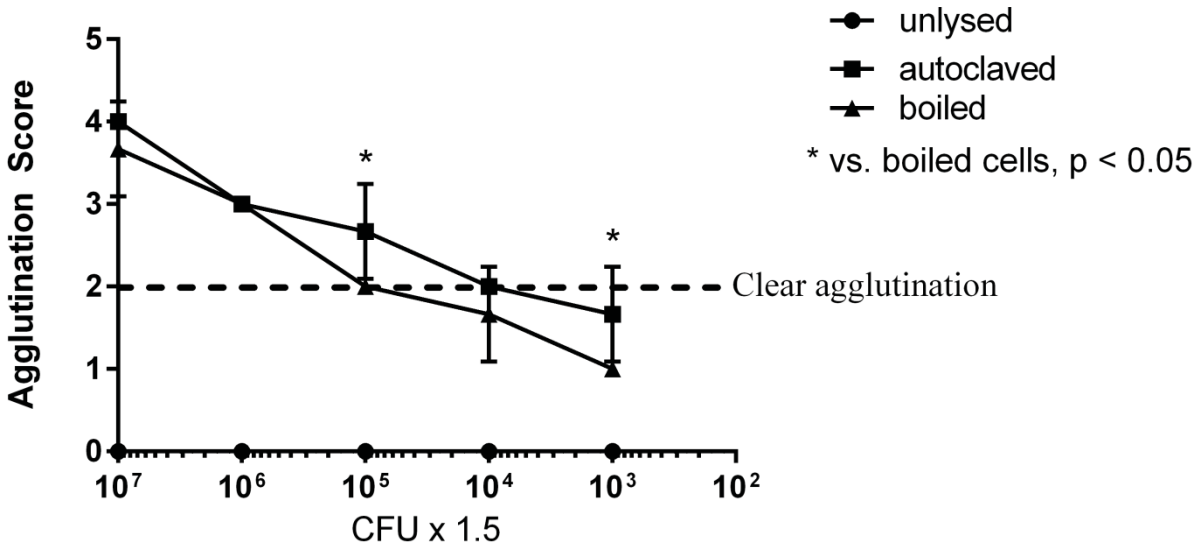

Figure 6. Agglutination of *H. parasuis* serotype 5 unlysed, autoclaved, or boiled cells. The agglutination reactivity was observed from the starting point of introduction of cells to 2 min after mixing. All lysed cells had significantly higher agglutination scores than unlysed cells ($p > 0.0001$), and autoclaved cells had significantly higher scores than boiled cells at concentrations of 1.5×10^5 and 1.5×10^3 CFU ($p < 0.05$).

3.4.5 Determination of SLP serotype specificity by PCR.

Thirty *H. parasuis* isolates of unknown serotype were grown on PPLO$^+$ agar, 10^6 CFUs were scraped off of the agar and boiled for 10 min, and cell debris was separated from the lysate by centrifugation. The lysates were resuspended and mixed with each serotype of SLP, and agglutination was scored after 2 min (Fig. 7). Cells from the same agar medium preparations were also harvested and lysed for DNA extraction. Multiplex PCR with primers for each serotype was performed on DNA from each clinical isolate and compared to the results from the reference serotypes. The multiplex PCR reaction with all three reference serotype strains was

confirmed by visualization under UV light, with bands for all three gene products in the multiplex reaction, and single bands for specific gene products in the multiplex reaction with one DNA sample per well (Table 3). Of the thirty isolates tested, 24 agglutinated with SLPs specific to serotype 4 strain SW124, which was confirmed by PCR. The remainder of the isolates agglutinated with SLPs specific for serotype 5 Nagasaki strain.

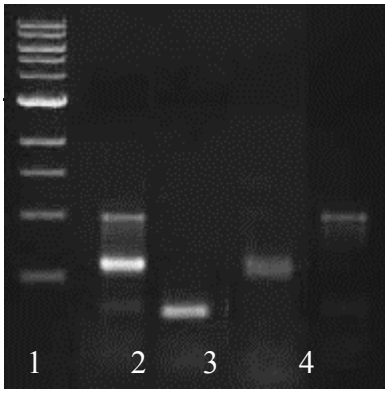

Figure 7. Multiplex PCR of reference serotypes. DNA extracted from *H. parasuis* serotypes 4, 5, and 9 were used in PCR with primers designed for genes in each capsule locus specific for each serotype. Lanes: 1- 1kb ladder; 2- serotype 4, 5, and 9 DNA with serotype 4, 5, and 9 primers; 3- serotype 4 DNA with serotype 4, 5, and 9 primers; 4- serotype 5 DNA with serotype 4, 5, and 9 primers; 5- serotype 9 DNA with serotype 4, 5, and 9 primers.

Table 3: Results of clinical isolate serotyping with SLPs and PCR.

Isolate	Serotype with clear SLP agglutination	PCR confirmation
SW124	4	Y
Nagasaki	5	Y
D74	9	Y
5013-96		N
41363	4	Y

W94-46357		N
15945-96	4	Y
46072	4	Y
42539	4	Y
16115-96	4,9	Y
4884-96	4	Y
10194-96	4	Y
W94-45548		N
W94-47951	4	Y
7033-96	4	Y
11524-96	4	Y
44510	4	Y
6249-96	4	Y
3034-96	4	Y
3548-96	4	Y
12783-96	4	Y
11166-96	4	Y
6901-96	4	Y
W94-46462	5	Y
W94-46463	5	Y
W95-572	4,9	Y
W94-45875	4	Y
W95-46072 S-9	4,9	Y
22309	5	Y

3.4.6 Serotyping of previously untypeable isolates

Six clinical isolates that either did not agglutinate with any serotype-specific SLP or agglutinated with multiple serotype-specific SLPs were re-evaluated for serotype specificity by agglutination of SLPs and by PCR following growth and lysis under different conditions. Bacteria were grown in broth for 24 h and were lysed by autoclaving, and the lysates were retested with the SLP assay. Three isolates did not agglutinate at all in the SLP assay, and their DNA were not characterized as any of the serotype-specific genes in PCR. Three of the isolates agglutinated with SLPs to both serotypes 4 and 9, despite dilution of the lysates past the point of clear agglutination. The PCR results for these isolates indicated that genes from both serotypes 4

and 9 were present in two of the isolates, and DNA from serotype 4 and a source that did not polymerize with primers for either serotypes 5 or 9 were present in the other isolate. These three isolates were re-cultured for colony isolation on agar medium, and cells from single colonies were examined by Gram-staining (Fig. 9A). The different colony types were sub-cultured until pure cultures were obtained, and the separated strains were re-assayed with the SLPs and their DNA was re-evaluated by PCR (Fig. 9B). Bacteria from all three cultures now agglutinated with serotype 4 SLPs, and two of the other colony types from the double serotype culture also agglutinated with serotype 9 SLPs. Of the 30 isolates of *H. parasuis*, 27 isolates were successfully typed with the SLP assay. These data were used to calculate the sensitivityof the SLP assay. The sensitivity of the SLP assay was 90.1%, with a 95% confidence interval of 0.7347 to 0.9789.

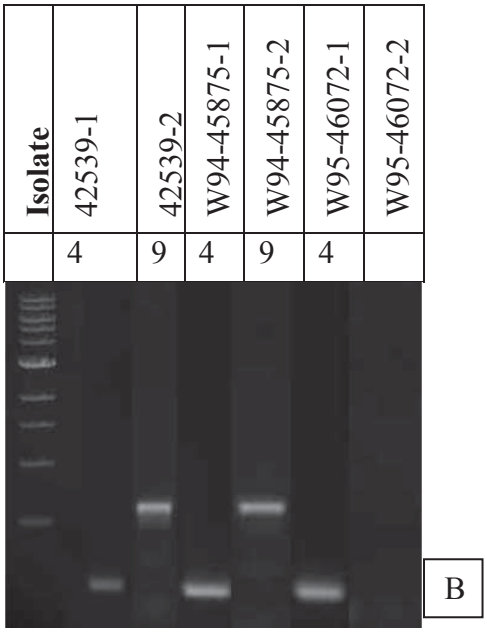

Figure 8. SLP and PCR serotype results of previously untypeable isolates. Cell morphologies (A) and isolated colony latex agglutination and PCR results (B). The three isolates that yielded mixed results in latex agglutination and PCR were re-cultured on agar medium and colonies of differing morphologies were isolated, re-cultured, and retested.

3.4.7 Determination of SLP specificity.

Twenty-seven swine bacterial isolates were grown on agar medium and lysed by boiling in the same manner as the *H. parasuis* isolates. The non-*H. parasuis* isolate lysates were used in the latex agglutination assay, and were also used in PCR with the *H. parasuis* serotype primers.

All 27 isolates were serotype negative in both the agglutination assay as well as in PCR (data not shown).

The data collected from the agglutination assays and PCR of the negative isolates were used to calculate specificity of the SLP assay, and the confidence intervals of the SLP assay were calculated using Fisher's exact test. The specificity of the assay was 100%, with a 95% confidence interval of 0.8389 to 1.000.

3.5 Discussion

When antibodies to purified *H. parasuis* CPs were conjugated to latex particles, they could be used in a rapid agglutination test for detection and serotyping of *H. parasuis*. This assay could be completed in two minutes and was more sensitive and specific than the current methods for typing. Latex agglutination assays have been developed for other Pasteurellaceae bacteria [8, 11], based on other bacterial subunits as well as CPs. The serotype-specific antigens of these pathogens had been well-defined before the creation of these assays. An indirect hemagglutination assay was previously developed for *H. parasuis* [12], using sheep red blood cells as the substrate for the antibody-antigen reaction for agglutination. However, several issues existed with this typing method, and therefore this method is not commonly used for diagnosis or serotyping. The assay took 2 h to complete, which is not efficient for high throughput processing in diagnostic laboratories. Reference serotypes were correctly identified by indirect hemagglutination, but only about 50% of clinical isolates were able to be serotyped accurately. Many isolates agglutinated with more than one serotype-specific antiserum, and the authors determined the serotype by choosing the strongest agglutination response. The *H. parasuis* cells used in typing were grown on agar medium and were not lysed, which may have contributed to

the lack of typing or mistyping of the isolates, as CP is not expressed on the cell surface of agar-grown cells [7]. Advances in serotyping of *H. parasuis* can be valuable for tracking strain migration through swine herds, as well as for developing an epidemiological scheme for prevalent strains and serotypes within a region, which would aid in rapid and effective diagnosis and treatment of infections.

The limits of detection for homologous serotypes by this latex agglutination assay were between 15-150 pg of purified CP depending on the specific serotype, and/or 1.5 x 10^5 CFU of broth-grown cells, or up to 1.5 x 10^4 from autoclaved cell lysates. These amounts can potentially be detected from biopsy or necropsy samples from pigs presenting with symptoms indicative of *H. parasuis* infection. Veterinary examinations and necropsies of symptomatic swine and pigs that have succumbed to infection demonstrate that *H. parasuis* can cause a broad spectrum of symptoms and pathologies, such as dyspnea, inappetance, paddling, fever, and swollen hocks with potential lesions on the skin, which are caused by pneumonia, pleuritis, pericarditis, meningitis, sepsis, and polyserositis [2, 13]. These symptoms and pathologies have many differential diagnoses, including Gram-Positive bacterial infections, enterobacterial infections, and even viral infections [14]. Therefore, diagnosis of *H. parasuis* infection is necessary in order to administer proper treatment to infected pigs. This diagnostic assay would eliminate the need to grow the samples in a diagnostic lab for *H. parasuis* detection, saving time and resources for the veterinarian and production farmer. However, due to lack of any clinical samples from infected swine, our assay was not able to be tested with clinical specimens. Nonetheless, a similar latex agglutination assay targeting *A. pleuropneumoniae* was able to detect CP in clinical samples [8], and therefore the same application should apply for the SLPs described here.

When CPs and cells were tested with each serotype-specific SLP, some agglutination was observed when using heterologous SLPs. Limited cross-reactivity of antiserum to purified *H. parasuis* CPs was also noted by ELISA [7]. The CPs of different *H. parasuis* serotypes share some of the same sugars [7, 15], which could explain the cross-reactivity with heterologous SLPs when higher concentrations of CP or cells are tested in assays capable of detecting very small quantities of CP. Therefore, it is important to note that higher concentrations of cells or purified CP may result in agglutination of heterologous serotype-specific SLPs and samples should be diluted appropriately before use in the SLP assay.

Because agar grown cells do not express CP [7], we lysed the bacteria grown on agar medium, which is a standard protocol for serotyping clinical isolates (J. Gallant, personal communication). Therefore, to assure that CP is present, the bacteria should be grown in broth medium first or adequately lysed prior to testing. We determined that boiling the bacteria yielded a slightly higher limit of detection than autoclaving the cells for 90 min, and the autoclaved bacterial lysate consistently had higher agglutination scores than the boiled cells. While boiling the cells is a more time-efficient method to lysing cells, some *H. parasuis* isolate bacteria that are boiled may not be able to be serotyped due to less available CP for agglutination, and autoclaving the cells for 90 min should be considered as a secondary method of confirmation.

The sensitivity and specificity of the SLP assay was 90.1% and 100%, respectively. Only three clinical isolates were unable to be serotyped using the assay or PCR confirmation. These isolates remain untypable, potentially due to the lack of appropriate antiserum or primers needed to identify the correct serotype. Serotypes 4 and 5 are the most prevalent types in our region, and most of the strains were identified as one of these two serotypes. Serotype 9 strains are considered to be avirulent, but were identified from the clinical isolates taken from symptomatic

swine. Our results indicated that multiple serotypes were capable of colonizing a host, and perhaps the competition between the two serotypes could trigger dissemination and establishment of infection. Further research is necessary to evaluate the prevalence of multiple serotypes present in swine before and during *H. parasuis* outbreaks, and if any cell-cell or host-cell signaling occurs for multiple serotypes to disseminate into the host. Even though our SLP assay could not correctly identify every positive sample, testing of other bacteria involved with differential diagnoses in swine yielded no false positive results. Current serotyping methods fall short of our achieved thresholds for sensitivity and specificity, with many strains being reported as either untypeable or of multiple serotypes (S. Brockmeier, V. Aragon, personal communications). Of significance is that one explanation for inaccurate serotyping may be the presence of multiple serotypes of *H. parasuis* or contaminants in the cultures, both of which were detected in some of our cultures. It should be emphasized that careful attention to uniform colony and cellular morphology is required before typing of isolates should be considered. *H. parasuis* is an opportunistic pathogen and may disseminate throughout the host after a prior infection [14]. If this infection is due to another bacterium, this agent may be co-cultured with *H. parasuis*. The Kielstein-Rapp immunodiffusion assay first used for *H. parasuis* serotype designation relies on the presence of a heat-stable antigen [5]. However, if only cell lysates are used in immunodiffusion assays for serotyping, the CPs from several strains of bacteria may still be present and the serotype may not be correctly identified, or cross-reactivity may occur, which could occlude the determination of the correct serotype. We were able to isolate multiple serotypes of *H. parasuis* from a single culture sample, and confirmed the presence of both by agglutination with the SLP assay and by PCR.

Currently, the latex agglutination assay is being optimized for greater sensitivity and specificity with the addition of SLPs conjugated to antibodies generated against other serotype-specific CPs. The assay will then be used test samples from infected swine, which will be required to determine the assay's potential for rapid diagnosis in real-time production swine health evaluations, as well as for appropriate serotyping after positive diagnosis of Glässer's Disease.

3.6 Acknowledgements

We thank Dr. Linda Zeller for providing the *H. parasuis* reference strains, and Dr. Karen Post for providing the clinical isolates for this study. We also thank Dr. Virginia Aragon, Dr. Susan Brockmeier, and Jackie Gallant for their technical and theoretical advice.

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Chapter 4

The Immune Response to and Protective Efficacy of a Novel *Haemophilus parasuis* Capsular Polysaccharide-Conjugate Vaccine for Glässer's Disease

Anne C. Michalenka Hyman[1]
Susan Brockmeier[2]
Crystal Loving[2]
Thomas J. Inzana[1, 3]

[1] Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA
[2] National Animal Disease Center, United States Department of Agriculture, Ames, IA
[3]Virginia Tech Carilion School of Medicine, Roanoke, VA

4.1 Abstract

Haemophilus parasuis is a Gram-negative bacterium responsible for Glässer's Disease in pigs, though little is known regarding its antigenic or virulence factors. Our goals were to isolate the capsular polysaccharide (CP) from *H. parasuis*, and determine its protective efficacy against Glässer's Disease in piglets. CP was purified and conjugated to the carrier protein Cholera Toxin B (CTB), and used to immunize mice and piglets before challenge with *H. parasuis* serotype 5 Nagasaki strain to evaluate the antibody response to *H. parasuis* CP. The specific humoral and cytokine responses to the CP and CP-conjugate were also more closely examined in immunized mice. Mice immunized with the CP-CTB conjugate and challenged with *H. parasuis* strain Nagasaki had significantly lower numbers of *H.* parasuis recovered from the blood and tissues than the unimmunized group inoculated with the same dose of bacteria. In mice, the CP-CTB conjugate produced significantly higher IgG_2 and Th_2 responses than the controls or mice immunized with CP alone, as determined by ELISA. However, there was also a significantly higher IL-2 response in the mice immunized with the CP-CTB conjugate as well, indicating a Th_1 response was induced in the CP-CTB immunized mice. In piglets, the CP-CTB conjugate produced a robust IgG, but significantly weaker IgM antibody response to CP, whereas the IgG response in unimmunized piglets did not significantly increase from the baseline antibody titers over the course of the study. The immunized piglets survived challenge with a high dose of *H. parasuis* serotype 5 Nagasaki strain, and exhibited less symptoms and bacterial presence in tissues than the unimmunized piglets ten days post-challenge. However, because 75% of the unimmunized piglets also survived and contained some antibodies to *H. parasuis* CP, the vaccine study remains inconclusive. Due to baseline antibody titers against CP that the piglets possessed before challenge, there is a possibility that a previous infection with *E. coli* conferred some

cross-protection. Nonetheless, the robust immune response to CP in piglets immunized with the CP-CTB conjugate provides optimism that a CP vaccine can be protective against *H. parasuis* infection. Repeated trials and refinement will be necessary to create an efficacious product.

4.2 Introduction

Haemophilus parasuis is a pleomorphic Gram-negative bacterium in the family Pasteurellaceae, and is responsible for Glässer's disease (polyserositis) in piglets and pneumonia in adult pigs. *H. parasuis* infects pigs worldwide, and is most commonly seen in animals from high-health or specific pathogen-free herds [1], or as a co- or secondary pathogen following previous viral or mycoplasma infection [2]. Infected pigs may develop polyserositis and arthritis, meningitis, pneumonia, or sepsis, and the disease can often be fatal, potentially killing sixty percent of a herd within 24-72 h [3]. Symptoms are generally observed in piglets 3 weeks to 4 months of age due to the stresses of weaning, relocation, or recovery from prior infection [4]. Currently, *H. parasuis* infections are treated with a course of β-lactam or fluoroquinolone antibiotics, but antibiotic-resistant strains are emerging globally [5]. Vaccines (bacterins or a live-attenuated strain) are available, but current vaccines do not protect against all serotypes [6], and the endotoxin present in whole cells can cause dangerous side effects [7]. Therefore a safer, cost-effective *H. parasuis* vaccine is needed to maintain optimum health in swine herds.

Fifteen *H. parasuis* serotypes have been identified globally [8]. Available vaccines are directed toward serotypes 4, 5, and 13 [9].The serotype-specific antigen has been recently identified as the capsular polysaccharide [10]. CP may contribute to bacterial virulence by promoting resistance to phagocytosis and complement-mediated killing [11]. As a result, antibodies to the CP may be opsonic and fix bactericidal complement, which would promote

clearance of the bacteria. The CP is the serotype-specific antigen in many genera of the Pasteurellaceae family, and antibodies to the CP are protective against disease by encapsulated, toxin-deficient bacteria [12, 13]. Therefore, the *H. parasuis* CP could be efficacious as a component in vaccines and in diagnostic assays. However, polysaccharides are generally poorly immunogenic and T cell-independent antigens that may stimulate only a transient IgM response [14]. This is especially the case for the CP of *H. parasuis*, which for many CP serotypes contains sialic acid, a glycose residue typically found on almost all eukaryotic cell surfaces, and is therefore not immunogenic [15]. Conjugation of a carrier protein has been shown to stimulate a T-dependent antibody response to polysaccharides, even in infants and young animals with an immature immune system [16]. We have confirmed that a CP is present on most *H. parasuis* isolates, but its expression appears to be regulated by environmental factors. Antibodies to the CP of *H. parasuis* can fix complement and mediate serum killing and clearance of bacteria *in vitro* [10], and therefore should be an important component in vaccines to protect against disease due to *H. parasuis* infection. We present preliminary evidence that the *H. parasuis* CP conjugated to a carrier protein induces a robust immune response in the host and may protect against Glässer's Disease.

4.3 Methods

4.3.1. Bacteria strains and growth conditions

The *H. parasuis* serotype 5 Nagasaki strain was kindly provided by Dr. Linda Zeller at the Iowa State School of Veterinary Medicine in Ames, IA. Bacteria were grown on either chocolate plates (Remel, Fair Lawn, NJ) or PPLO agar medium (BD, East Rutherford, NJ) supplemented with 1% glucose (Sigma, St. Louis, MO), 5% fetal bovine serum (FBS, Atlanta

Biologicals, Norcross, GA), and 100 µg/ml nicotinamide adenine dinucleotide (Acros Organics, Geel, Belgium) ($PPLO^+$). For bacterial challenge in piglets, cells were grown on solid agar medium at 37°C for about 48 h and diluted to the challenge concentration of 10^4 colony forming units (CFU)/piglet, which was determined spectrophotomerically. For CP purification, bacteria were incubated on solid agar medium at 37°C for about 48 h, inoculated to 20 ml of $PPLO^+$ broth, and shaken at 37°C until mid-log phase, or 10^9 CFU/ml), which was determined spectrophotometrically by Klett meter and confirmed by viable plate count. The 20 ml culture was used to inoculate 2 L of $PPLO^+$ broth, which was shaken at 37°C until the culture reached early stationery phase, roughly 72 h.

4.3.2 Capsular polysaccharide purification

CP was purified from *H. parasuis* using methods previously described [10]. Briefly, bacteria were grown in $PPLO^+$ broth to early stationery phase, and sedimented by centrifugation at 10,000 x *g*. The supernatant was saved, and the pellet was resuspended in 50 ml of distilled water, heated at 121°C for 30 min, and centrifuged as above. The supernatant was treated with Cetavlon (Sigma), enzymatic digestions to remove nucleic acids and proteins, and then treated with 45% phenol. The phenol phase was separated from the aqueous phase by centrifugation. The aqueous phase was removed, distilled water added to the phenol phase, and the extraction repeated until no proteinaceous material was present at the water-phenol interface. The aqueous phases were pooled, dialyzed against distilled water, and LOS in the samples removed by centrifugation at 41,000 x *g* for 4 h at 4°C. Final purification was by gel filtration chromatography (Sephacryl S-400; GE Healthcare Life Sciences, Piscataway, NJ).

Carbohydrate-positive fractions [17] that eluted in the void volume were pooled, dialyzed, and lyophilized.

4.3.3 Antisera to CP

New Zealand White rabbits were immunized with CP as described previously [10]. Rabbits were exsanguinated when antibody titers to CP were greater than 1:10,000, as determined by ELISA. Antisera were stored at -20°C until needed.

4.3.4 Conjugation of CP to carrier protein

Ten mg of CP was suspended in 1 ml of 0.1 M sodium acetate buffer, pH 5.5 (Sigma), and then mixed 1:1 with 20 mM sodium *m*-periodate (Sigma) in 0.1 M sodium acetate buffer. The mixture was incubated on ice for 30 min in the dark. The oxidized CP was dialyzed against PBS overnight to remove excess periodate, and the structural integrity of the CP was evaluated by ELISA. The CP was then added to solubilized hydrazide-streptavidin (ThermoPierce, Fairlawn NJ) at 5 molar excess to the streptavidin, and mixed end-over-end for 2 h at room temperature. The conjugation of streptavidin to the CP was measured by sandwich ELISA, using anti-CP antiserum CP for capture and HRP-tagged biotin as the reporter conjugate (see below). The CP-streptavidin intermediate was then added to biotinylated CTB and the mixture incubated at room temperature overnight, rotating end-over-end. The CP-CTB conjugate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Blue staining.

4.3.5 ELISA

Direct ELISAs were performed to determine the integrity of the oxidized CP before conjugation to streptavidin, or to evaluate mouse or swine antibody response to CP or CP-CTB. For each ELISA, samples were plated in triplicate and each ELISA was repeated at least three times. Ten μg/ml of purified CP, oxidized CP, or CP-CTB, or 10^6 CFU of *H.* parasuis serotype 5 Nagasaki strain suspended in PBS supplemented with 2 mM $MgCl_2$ (PBS-M, Sigma) were added to Immulon 4HBXmicrotiter plate wells (Immulon, Fair Lawn, NJ). All incubations were carried out at 37°C for 1 h, and the plates washed at least 3 times with PBS with 0.05% Tween-20 (Fisher) (PBST). Nonspecific binding was blocked with 2% nonfat dry milk (NFDM) in PBS, the plates were washed, and incubated with serial dilutions of anti-CP serum generated from rabbits, mice, or swine in 2% NFDM in PBST. The plates were washed, and horse radish peroxidase (HRP)-tagged anti-rabbit IgG (Jackson Immuno, West Grove, PA), anti-mouse IgM or IgG_{h+l}, (Jackson Immuno), or anti-swine IgG (Kirkegaard and Perry Labs, Gaithersburg, MD) or IgM (kindly given by Drs. Susan Brockmeier and Crystal Loving from the National Animal Disease Center, Ames, IA) diluted 1:5000 in PBST was added. After thorough washing, 3,3',5,5'-tetramethylbenzidine (TMB)-peroxidase (ThermoPierce) was added until color developed. The reaction was stopped with 1 M H_2SO_4 and the A_{450} determined.

Sandwich ELISAs were used to measure the amount of streptavidin conjugated to CP, as well as determine the amount of IgG_1 and IgG_2 present in mice immunized with CP or CP-CTB. Hyperimmune rabbit antiserum to CP (diluted 1:100 in PBS-M), or antiserum to mouse IgG_1 or IgG_2 (diluted 1:500) was added to a high-binding plate. All incubations and washes were carried out as in direct ELISA. For determining the amount of CP conjugated to streptavidin, after blocking nonspecific binding, as above, the plates were incubated with 100 μg of either CP,

ADH-streptavidin (as a negative control), or CP-streptavidin in 2% NFDM in PBST. The plates were washed, and HRP-tagged biotin diluted to 1:5000 was added and plates were incubated for 1 h. After washing, TMB-peroxidase was added until color developed, and the reaction was stopped with 1 M H_2SO_4 and the A_{450} determined. For determining IgG_1 and IgG_2, after nonspecific binding was blocked, plates were incubated with sera from mice immunized with control PBS, CP, or CP-CTB in 2% NFDM in PBST. The plates were washed, and incubated with HRP-tagged anti-mouse IgG (Jackson Immuno) diluted to 1:5000. After the final washing, TMB-peroxidase was added until color developed, and the reaction was stopped with 1 M H_2SO_4 and the A_{450} determined.

The quantification of IFN-γ, IL-2, IL-4, and IL-10 cytokines was performed using a mouse Th_1/Th_2 Ready Set Go! ELISA kit (eBioscience, San Diego, CA), following the manufacturer's protocol.

4.3.6 Immunization of mice to evaluate the immune response to CP-CTB

Eight Swiss Webster mice (Harlan, Indianapolis, IN) were used to determine the specific IgG antibody and cytokine response. Two mice were immunized with only PBS. Three mice were injected subcutaneously (SQ) with 0.1 ml of 50 µg of serotype 5 CP in PBS mixed 1:1 with Freund's Incomplete Adjuvant (Sigma). The remaining mice were inoculated with 50 µg of CP-CTB, also in PBS mixed 1:1 with Freund's Incomplete Adjuvant. Mice were boosted SQ with the same PBS, CP or CP-CTB formula after 21 d, and then intraperitoneally with CP or CP-CTB in sterile PBS every 10-14 d for a total of four times in six weeks, with the last immunization three days prior to bacteria challenge. Test bleeds were taken from the submandibular vein every 10-14 days post-immunization to evaluate antibody titers. Mice were euthanized and

exsanguinated at the end of the study, and blood samples to assay for cytokines were stored immediately in liquid nitrogen. Sera from all collected blood samples were stored at -20°C until used.

4.3.7 Protective efficacy of CP antibodies in mice

Eight Swiss-Webster mice were divided into four groups, with two groups receiving an intraperitoneal dose of 10^7 - 10^8 CFU *H. parasuis* Nagasaki strain mixed with 2% (v/v) hog gastric mucin [18], and two groups receiving an intranasal dose of 3 x 10^7 – 3 x 10^8 CFU *H. parasuis* Nagasaki strain in sterile PBS. The mice were monitored daily for health status and were euthanized after 72 h, or when animals became moribund. Liver, lung, and spleen tissue sections were taken during necropsy, homogenized with PBS supplemented with $CaCl_2$ and $MgCl_2$, spread onto $PPLO^+$ agar medium, and incubated for 48 h at 37°C. The highest dose of *H. parasuis* that yielded bacterial presence in tissues was the intraperitoneal dose of 10^8 CFU, and that concentration was used to challenge mice immunized with PBS, CP, and CP-CTB as described above. Following challenge the immunized mice were monitored for health status, and were euthanized after 96 h or when animals became moribund. Tissue sections from liver, lung and spleen were recovered during necropsy, homogenized, and cultured on $PPLO^+$ agar medium to determine bacterial loads.

4.3.8 Protective efficacy of CP antibodies in piglets

Eight Large White x Landrace caesarian-derived, colostrums-deprived (CDCD) piglets were split into two groups: a control group received intramuscular doses of sterile saline during each round of inoculation, and an immunization group received 100 µg of CP-CTB conjugate in

sterile saline supplemented with Emulsigen-D adjuvant (MVP Technologies, Omaha, NE). Blood was collected from each piglet to determine the antibody response to CP-CTB before each immunization, before bacterial challenge, and at the end of the challenge study. Also prior to the study, the nasal cavities of each pig were swabbed, immersed into 1 ml PBS, and 100 µl were spread onto chocolate agar plates and incubated at 37°C for 48 h to determine if *H. parasuis* was present in the animals prior to the study. Piglets were immunized on day 0, boosted on day 21, and challenged on day 54. All animals received 10^4 CFU of *H. parasuis* serotype 5 Nagasaki strain intranasally. Animals were monitored daily for changes in health, and were humanely euthanized either when they became moribund or at day 62. At necropsy, swabs from the nasal cavity, lung , serosa (pericardium, pleura, peritoneum), and hockjoint were extracted into 1 ml of PBS, and 100 µl of each sample was spread onto chocolate agar, as well as 100 µl each of cerebral spinal fluid and serum. The agar plates were incubated for 48 h at 37°C, and colonies were counted and assessed for the presence of *H. parasuis* serotype 5. Antibody titers were determined by ELISA using serotype 5 CP and CP-CTB as antigen.

4.3.9 Data processing and statistical analyses

Student's t-test and two-way ANOVA with Bonferroni post-test calculations were determined using InStat GraphPad Prism v. 6.05 to determine statistical significance. Scatterplots, histograms and survival curves were also generated with GraphPad Prism.

4.4 Results

4.4.1 Conjugation of CP to carrier protein

The serotype 5 CP when conjugated to hydrazide-streptavidin maintained antigenic integrity, determined by sandwich ELISA (Fig. 1A). The CP-streptavidin mixed with biotinylated cholera-toxin B resulted in successful conjugation that was maintained during SDS-PAGE, as confirmed by Coomassie-blue staining (Fig. 1B).

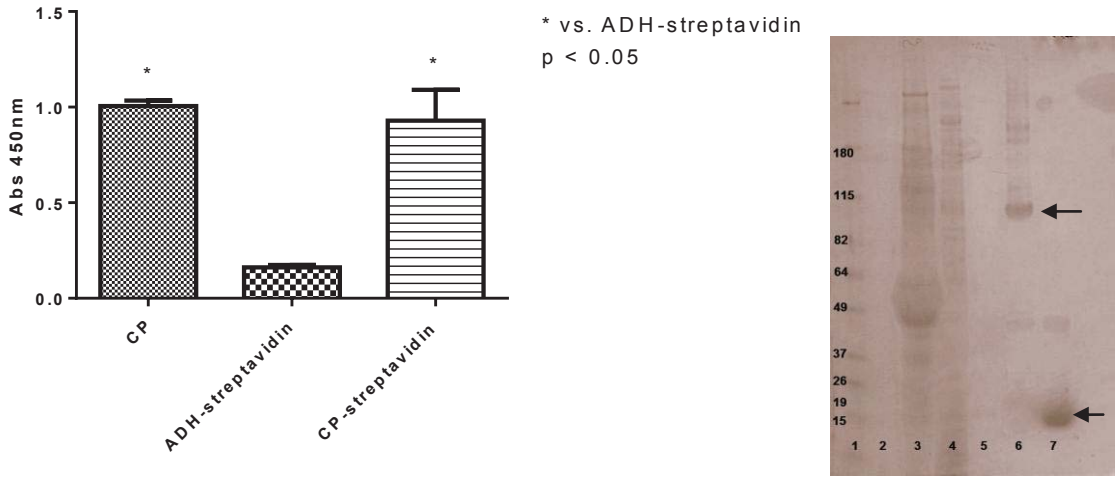


Figure 1: Integrity of CP after conjugation to ADH-streptavidin (A) and to biotinylated cholera toxin B (B). CP, ADH-streptavidin, and the CP-streptavidin intermediate were used as captured antigens in a sandwich ELISA. The CP-CTB conjugate was electrophoresed on a 4%/8% bis/tris gel, and stained with Coomassie Blue, resulting in an upward shift in protein size when conjugated. Lanes: 1, molecular size standards; 2, no material; 3, CP-ADH-streptavidin, 4-ADH-streptavidin, 5- CP, 6- CP-CTB (indicated by arrow), 7- biotinylated CTB (indicated by arrow).

4.4.2 Immune response to CP-CTB by mice

Mice immunized with CP alone or the CP-CTB conjugate developed a combined total IgG and IgM response to CP, whereas the mice that were not immunized did not generate a significant antibody response to CP (Fig. 2). Mice immunized with CP alone made a

significantly greater total IgG and IgM response to CP than CP-CTB ($p < 0.05$). However, the mice immunized with CP-CTB had a significantly more robust total IgG and IgM response to CP-CTB than the mice immunized with CP alone ($p < 0.01$).

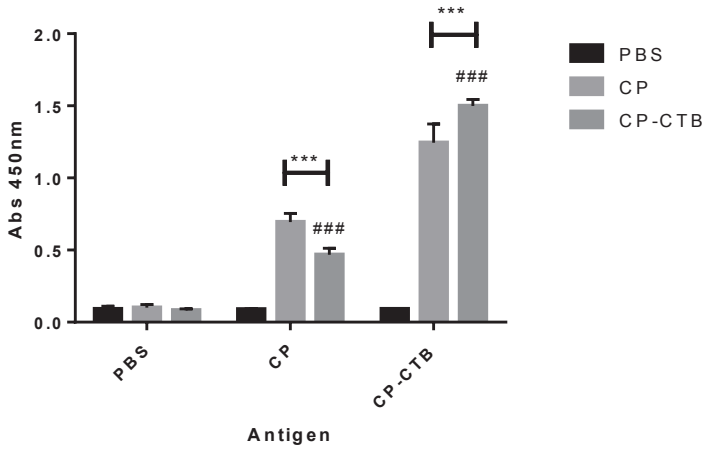


Figure 2. Total IgG and IgM response to CP and CP-CPB in mice. Sera were taken from mice immunized with either sterile PBS, CP or CP-CTB to assess antibody titers to the specific antigens by ELISA at 1:1000 concentration of antisera. ***- vs. PBS response, ###-vs. antibodies made to CP.

Murine IgM-, IgG_1-, and IgG_2-specific antibody responses to CP and CP-CTB were determined by ELISA (Fig. 3). All immunized mice produced a significantly higher IgG_1 and IgG_2 response than IgM after immunization (Fig. 3A) than the pre-immunization bleed ($p < 0.001$). Unimmunized mice did not produce an antibody response over the course of immunization (data not shown). Mice immunized with CP had significantly higher amounts of IgG_2 from immunization until the second to last bleed before challenge, whereas mice immunized with CP-CTB had significantly higher amounts of IgG_2 throughout the course of the immunization study.

At the time of bacteria challenge (Fig. 3B), all mice made a significantly weaker IgM response to CP and CP-CTB than to either IgG-specific response ($p < 0.001$). The antisera derived from mice immunized with PBS did not produce any significant antibody response. Mice immunized with CP had significantly higher levels of IgG than IgM ($p < 0.001$) but produced levels of IgG_1 and IgG_2 that were not significantly different. However, mice immunized with CP-CTB also had a significantly weaker IgM response than either IgG type ($p < 0.001$), but had a significantly more robust IgG_2 response than IgG_1 ($p < 0.001$).

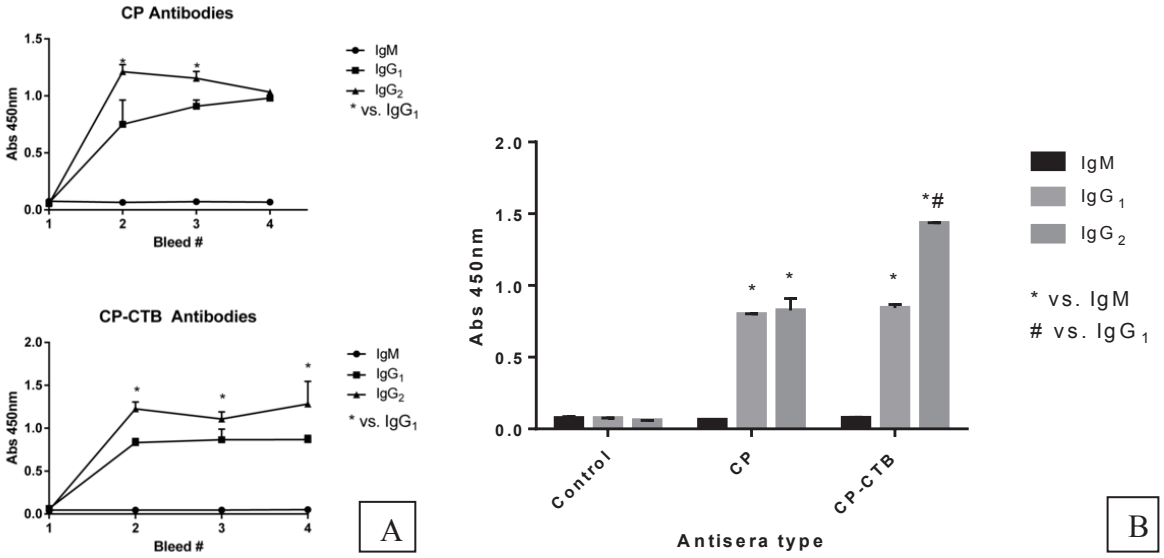


Figure 3. Antibody response to CP and CP-CTB in mice throughout immunization (A) and before challenge (B). A, Antibody levels for IgM, IgG_1 and IgG_2 from every bleed during the course of immunization were determined by ELISA. B, Antibody titers from final bleed before bacterial challenge.

Sera obtained from mice at the end of challenge were used at 1:1 concentration in ELISA to determine levels of IFN-γ, IL-2, IL-4 and IL-10 in response to *H. parasuis* challenge in each of the immunization groups (Fig. 4). There was no significant difference in IFN-γ levels between immunization groups. Mice that were immunized with CP did not have significantly higher

levels of cytokines than unimmunized mice. However, mice immunized with CP-CTB had significantly higher levels of IL-2, IL-4, and IL-10 than the other mouse groups ($p < 0.01$ for IL-4, $p < 0.001$ for IL-2 and IL-10). The IL-2 and IL-10 cytokine amounts were significantly higher than the IL-4 response in the CP-CTB immunized mice ($p < 0.001$).

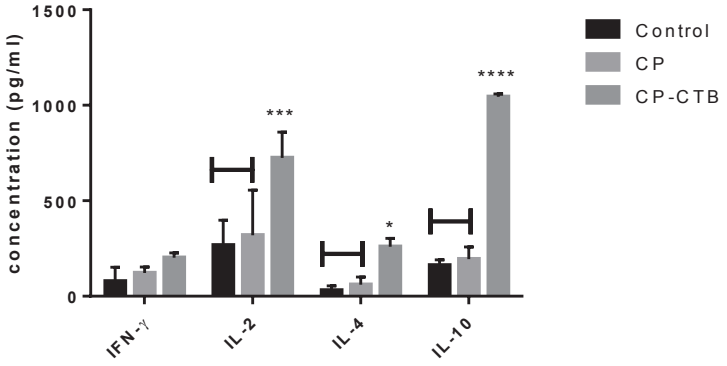


Figure 4. Cytokine response of mice to *H. parasuis* infection. Cytokine amounts from each immunization group were determined by ELISA.

4.4.3 Protective efficacy of the CP-CTB conjugate in mice

All immunized mice were challenged with 10^8 CFU of *H. parasuis* Nagasaki strain intraperitoneally with 2% hog gastric mucin. While none of the mice from either immunization group became moribund, the unimmunized mice exhibited dehydration, inappetance, and sluggishness 12 h post-challenge and recovered slowly to the challenge endpoint. All mice were bled at 12 h and sera were cultured on agar medium to evaluate the mice for bacteremia. No bacteria were recovered from sera from immunized mice12 h post-challenge where roughly 30 CFU was recovered from 20 µl of sera from unimmunized mice (data not shown). At necropsy, unimmunized mice had significantly higher *H. parasuis* loads in all tissues compared to the

immunized mice. There was no significant difference in the number of bacteria present in tissues between the immunized groups.

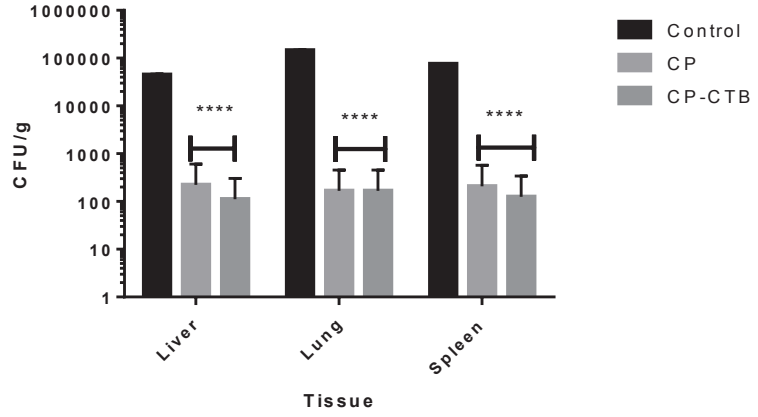


Figure 5. Bacterial CFUs in tissues of challenged mice. Tissues were extracted from immunized and non-immunized mice challenged with *H. parasuis* Nagasaki strain, and cultured on agar medium to determine bacterial colony forming units (CFUs).

4.4.4 Protective efficacy of the CP-CTB conjugate in piglets

Eight piglets, divided into control and immunization groups, were either immunized with sterile saline or the CP-CTB conjugate with Emulsigen-D adjuvant, and were boosted over the course of six weeks before challenge with *H. parasuis* Nagasaki strain. One piglet from the control group succumbed to disease caused by *H. parasuis* infection before the end of the study and was euthanized (Fig. 6). The remaining pigs in both groups did not exhibit substantial symptoms related to infection or disease. Necropsy results yielded pathologies found in the lung, pericardium, and pleura in the unimmunized animals, and on the pericardium and pleura of two of the immunized pigs. Three of the unimmunized animals had *H. parasuis* present in the nasal turbinates, lungs, sera, and CSF, while two of the immunized animals had bacteria present in the

nasal turbinates, lung, and CSF. All piglets with *H. parasuis* present in the lungs had over 100 CFU per swab (Table 1).

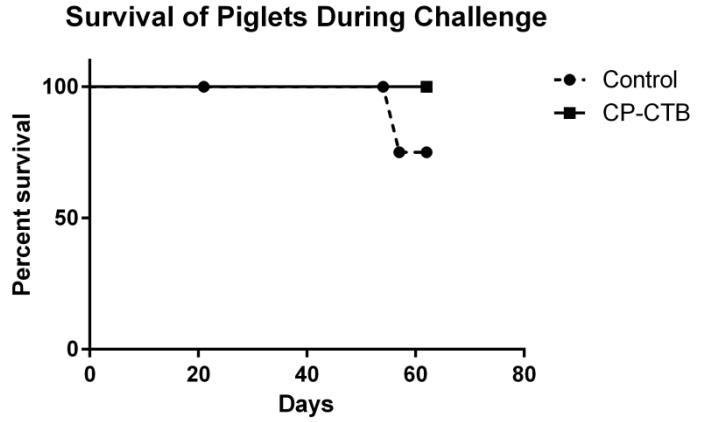


Figure 6. Survivial of piglets following immunization with CP-CTB and *H. parasuis* challenge. Piglets were either immunized with sterile saline or with the CP-CTB conjugate, and challenged with *H. parasuis* Nagasaki strain. The health status for each pig was monitored and pigs were euthanized when they became moribund or at the end of the challenge study.

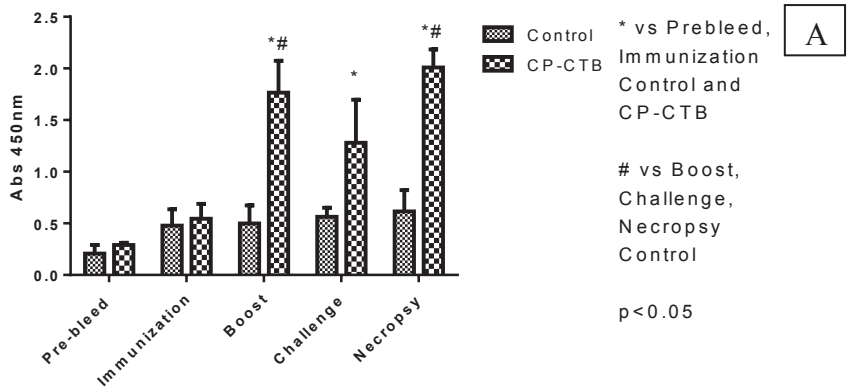
Table 1: Necropsy results of swine challenged with *H. parasuis*.

Pig	Symptoms	Diagnoses	Tissues with lesions	Samples with *H. parasuis*
Ctrl 1	Paddling, lethargy	Neuragia	lung (minimal)	nasal++, lung++, CSF++, serum
Ctrl 2	none			
Ctrl 3	none	serositis	pericardium, pleura	nasal+, lung++
Ctrl 4	none		lung (minimal)	nasal++ (contamination)
CP 1	none		pleura (minimal)	
CP 2	none			
CP 3	none	serositis, pneumonia	pericardium, pleura	nasal, lung++, CSF
CP 4	none	serositis	pleura	nasal++ (contamination), lung+

[a] CFU amounts: (no indicator) = <100, + = 100 < CFU > 200, ++ = too many to count

4.4.5 Immune response to CP-CTB conjugate in piglets

Serum samples from each timepoint for each pig were evaluated for antibody reactivity to either CP or the CP-CTB conjugate (Fig. 7). Most of the piglets had an immune response to the CP, even before immunization. This baseline response did not significantly increase in the unimmunized pigs during the study. The one piglet that did not have a baseline response to CP or CP-CTB succumbed to *H. parasuis* infection on day 57. However, the piglets immunized with CP-CTB had a significantly greater reactivity to CP than the control piglets after the initial immunization for the rest of the test bleeds, and immunized piglets showed significantly greater reactivity to CP after immunization than their baseline test bleeds. The pre-immunization and challenge sera from control piglets had minimal reactivity to the CP-CTB conjugate, even though the antibody titers were significantly higher at challenge ($p < 0.05$). However, the antibody response of the immunized piglets just before challenge was significantly greater than the response at the time of the initial immunization, and was significantly greater that the response of the unimmunized piglets at challenge ($p < 0.001$).



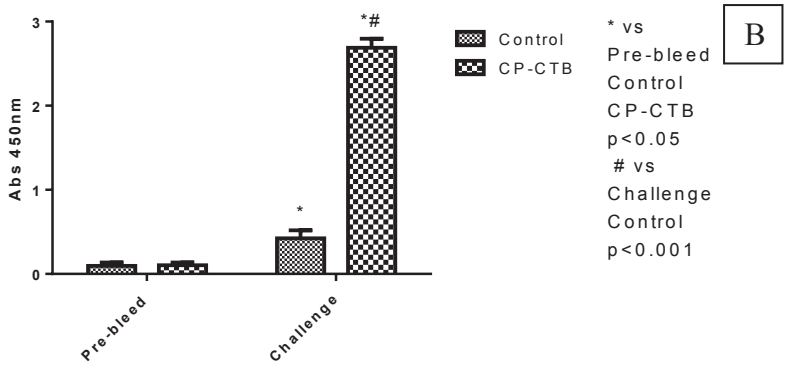


Figure 7: Reactivity of control and immunized pigs to CP (A) and the CP-CTB conjugate (B). Piglets were bled and immunized as described in Methods. Serum samples obtained prior to the initial immunization and at challenge were tested by ELISA to determine the antibody response to the CP and CP-CTB conjugate. Although the control piglets developed a significant antibody response before challenge compared to pre-immunization ($p < 0.05$), the CP-CTB-immunized piglets created a much more significant antibody response to the conjugate ($p < 0.001$).

Sera from all piglets were used to determine the IgM and IgG responses to CP-CTB by ELISA (Fig. 8). The IgM response across all groups did not change significantly over the course of immunization and challenge. The IgG response did not significantly increase over the course of the study in the control piglets. However, in the piglets immunized with the CP-CTB conjugate, the IgG response increased significantly over the course of immunization and challenge, and was significantly greater than the IgM response.

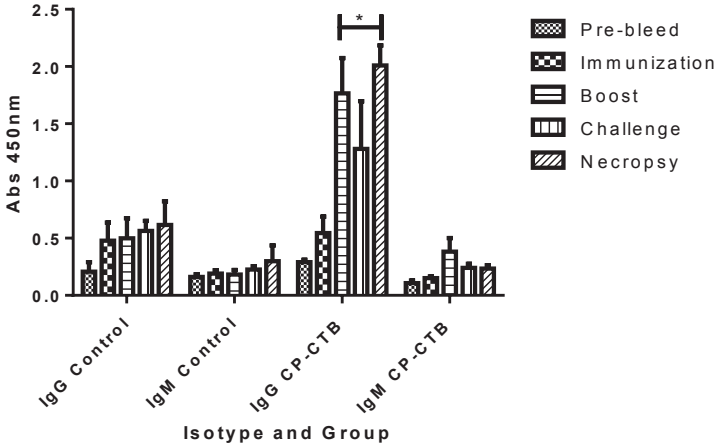


Figure 8. Comparison of IgM and IgG responses to the CP-CTB conjugate. IgM and IgG levels from each serum sample were determined by ELISA.

4.5 Discussion

H. parasuis serotypes produce CPs that are the predominant serotype-specific antigens of each serotype, and they contribute to virulence. The amount of CP produced and expressed on the cell surface is dependent on environmental factors, such as broth or agar medium, and the presence of bicarbonate [10]. Growth in broth may mimic physiologic systemic conditions during dissemination [19], and CPs of *H. parasuis* have only been observed in clinical isolates taken from systemic locations [20]. The current understanding of *H. parasuis* pathogenesis is that the bacteria live commensally in the oropharynx in pigs, but an unknown mechanism may enable the cells to disseminate and cause widespread, multi-tissue infection [3, 21]. The surface expression of CP would be required to protect *H. parasuis* in the bloodstream and tissues by evading opsonization and bactericidal factors. The presence of sialic acid as one of several glycose residues in the CP [22] may also contribute to evading detection by the immune system, as sialic acid is a normal component of host tissues, and is recognized as "self" [15].

The discovery of CP as the antigenic determinant and a virulence factor of *H. parasuis* is important, as little was previously known regarding the role of CP in virulence and type specificity [22]. Many genera of the Pasteurellaceae family produce CPs, and in many cases antibodies against CPs from non-toxin producing bacteria are protective against infection [23]. The identification and characterization of CP can lead to potentially safer treatments and preventative measures for production swine.

We report the novel utilization of the highly covalent bond between streptavidin and biotin to conjugate the CP of *H. parasuis* to a carrier protein. Subunit vaccines comprised of proteinaceous material [24] or conjugated to a carrier protein [25] are known to produce robust immune responses in the host. Outer membrane protein subunit vaccines have been designed to protect against *H. parasuis* infection [26], however the protective efficacy of the candidates fell short of consistent and total protection against infection. Perhaps the proteins are immunogenic, but are being shrouded by CP when the bacteria disseminate, or the CP prevents phagocytosis and peptide processing for presentation to T cells by macrophages. The CP conjugate vaccine not only preserves the structural integrity of the polysaccharide epitopes for antibody recognition, but the protein component also activates the adaptive immune response.

Mice were used in this study to evaluate the immune response to CP, when used with adjuvant alone or conjugated to a carrier protein. While not an effective model to evaluate Glässer's disease [27], mice can produce a specific immune response that can be determined using widely available commercial products. For effective protection against bacterial infection, a Th_2 response which generates antibodies against non-protein molecules is necessary, but in some cases not sufficient to effectively clear the pathogen from the host [28, 29]. The mice immunized with CP-CTB created a more robust IgG_2 response than IgG_1, even though both

antibody types are made in response to immunization. When activated and differentiated, IgG_1 and IgG_2 can neutralize pathogens, promote opsonization, and activate the compliment system, but IgG_2 is much more potent in its effector functions also promotes localized inflammation [30]. The CP-CTB immunized mice also developed a stronger response to IL-2 and IL-10 than IFN-γ and IL-4 than unimmunized mice or CP-immunized mice. These results indicate both Th_1 and Th_2 responses were made to the conjugate [31]. IL-10 suppresses IFN-γ, which could explain the low quantities of that cytokine [32]. The use of CTB as the carrier protein may have assisted in creating the balanced Th response [33]. The balanced adaptive and humoral responses indicated that a robust immune response was generated when animals were vaccinated with the CP-CTB conjugate, thus further evaluation of the vaccine product in piglets is needed.

While the CP-CTB conjugate produced an antibody response in piglets, it remains inconclusive as to whether the antibody response produced against CP is adequate to confer protection against Glässer's Disease. Several mitigating factors could potentially explain these inconclusive results. The unexpected survival of control animals after a lethal dose of *H. parasuis* was administered could be due to the caesarian-derived piglets becoming infected with an antibiotic-resistant strain of *E. coli*, due to potential cross-protection afforded by the CP of that strain. As a result of the *E. coli* infection, thirty-two animals needed to be euthanized prior to the study. The remaining animals were examined and deemed healthy enough to be placed in a smaller pilot study to determine the *in vivo* antibody response to CP and potential protection against a lethal dose of *H. parasuis*. However, it is unclear whether the animals were tested for residual *E. coli* presence at the time of immunization, or what strain of *E. coli* was responsible for prior infection. While the CP-CTB-immunized piglets had a robust antibody response to CP and the conjugate, the control animals also had a weaker, but present antibody response to the

same CP. The antibody response to CP in three of the control animals increased after immunization until the end of the study. The possibility of cross-reactive antibodies produced against an *E. coli* antigen is a potential explanation [34]. The CP locus of *H. parasuis* has strong homology to the type I CP locus of *E. coli*, but it is still unknown if similar sugars to the *H. parasuis* CP existed in the strain of *E. coli* that affected the facility. However, we remain optimistic that the immune response generated in the piglets that received the CP-CTB vaccine could be protective against *H. parasuis* infection.

4.6 Acknowledgements

We thank Dr. Linda Zeller for providing the reference strains of *H. parasuis*, the Teaching and Research Animal Care Support Service at the Virginia-Maryland Regional College of Veterinary Medicine for their exemplary care of the mice used in this research, and the animal support staff of the National Animal Disease Center for their care of the piglets used in this research.

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Chapter 5

Future Directions

Anne C. Michalenka Hyman[1]

[1]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia, USA.

5.1 Proposed experiments

This dissertation research has demonstrated that the capsular polysaccharide of *H. parasuis* is the serotype-specific antigen, but that its expression is regulated depending on environmental factors. The CP contributes to virulence by making bacteria resistant to serum-mediated killing unless in the presence of antibodies generated against CP. Antisera derived against CPs can also be used successfully in rapid identification and serotyping of *H. parasuis* from infected animals. When CP is conjugated to an immunogenic carrier protein, a robust immune response can be generated in the host animal. However, further refinement of experimental conditions is needed to conclude that the vaccine candidate is effective in preventing Glässer's Disease. Further experiments will be useful in identifying the role of CP expression in pathogenesis and expanding upon the use of CP in creating detection and prevention tools to bolster swine herd health. These experiments can be broken down into four studies: 1) Expansion of the understanding the regulation of the CP locus; 2) Studies evaluating bicarbonate levels in *in vitro* and *in vivo* models; 3) Refinement and packaging of the latex agglutination assay; and 4) Re-evaluation of the swine model and expanding on the spectrum of protection of the CP-CTB conjugate.

We have determined that the CP of *H. parasuis* is produced, but not expressed constitutively on the cell surface, and its expression relies on environmental conditions [1]. The genes in the CP locus are upregulated in the presence of broth medium and also bicarbonate, especially *iscR*, a putative regulatory gene at the end of region I [2]. RNA-sequencing analysis of bacteria grown on agar and broth with or without bicarbonate supplementation will allow us to identify other regulatory factors found genome-wide that influence CP expression. Once other potential genes are identified, the target proteins can be purified and used in electrophoretic

mobility shift assays [3] to determine if the proteins are responsible for promoting or inhibiting the expression of the CP locus. Finally, mutagenesis of specific genes using a previously developed method in *H. parasuis* [4-6] to knock out individual genes in the CP locus or other targets (such as *iscR*), would allow us to more accurately define the role of CP in pathogenesis and virulence. Mutated strains would be evaluated for CP expression under the same growth conditions used in the expression and type specificity studies and immunoassays. If shown to be attenuated in virulence, the CP-deficient mutants would be compared to our CP-CTB vaccine candidate as well as commercially available vaccines to determine the optimal source for protection against Glässer's Disease [7].

Bicarbonate is an important molecule in the expression of CP in *H. parasuis*, but further analysis of the biochemistry involved is necessary to understand bicarbonate's regulatory contribution. We know that bicarbonate is a molecule utilized by the host to buffer systemic stress responses [8], and bicarbonate is a useful source of carbon dioxide for bacteria [9]. The presence of bicarbonate during *H. parasuis* infection, either as a positive feedback autocrine molecule, or produced by host cells should be determined by monitoring bicarbonate levels in growth cultures, co-culture experiments, and *in vivo* during challenge studies. Bicarbonate is only one known source to influence cell signaling pathways that influence gene regulation [10]. Iron influences capsule production in the yeast *Cryptococcus neoformans* [11]. The role of iron should also be studied in a similar fashion as bicarbonate, especially since iron regulation is the function of iscR in *E. coli* [12].

The latex agglutination assay will be effective at identifying *H. parasuis* responsible for infection, and for rapid serotyping of isolates, particularly those that were previously untypable, now that the serotype-specific antigen has been confirmed. Creation of SLPs conjugated to all

anti-CP antibodies from each serotype would be beneficial for serotyping and tracking of all virulent and avirulent strains. We have recently acquired another thirty strains from the Aragon lab in Spain [13-16] that have been deemed untypeable, and they will be evaluated with our current SLP assay. Future studies include use of the SLPs to evaluate the sensitivity and specificity of the assay in diagnosing *H. parasuis* infection from freshly acquired tissues, fluids and fibrin from examination and necropsy of compromised animals. Once similar levels of sensitivity and specificity to the laboratory assay have been reached with field samples, the focus will shift to creating a packaged product for veterinarians for field trials for diagnosis, after evaluating the assay with the inclusion of inert preservatives for SLPs for field veterinary diagnostic kits.

Finally, repeating the protective efficacy study using the CP-CTB conjugate is necessary to determine if piglets can be effectively vaccinated with this subunit compound. If the trial is successful, multivalent CP-CTB conjugates using CPs derived from all known virulent serotypes [15, 17, 18] will be evaluated for their levels of protection against known strains, as well as blind studies. If successful, the vaccine will be packaged and used in larger studies with susceptible herds.

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