RESEARCH REPORT
SWINE HEALTH

Title: Molecular Characterization and Protective/Diagnostic Application of the Capsular Polysaccharide of Haemophilus parasuis- **NPB #12-014** revised

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Industry Summary

Haemophilus parasuis (Hps) is an important swine pathogen that can decimate a swine herd in less than 72 hours. Although this bacterium has devastating effects on pork producing facilities, little is known about how the bacteria infect pigs, and even less is known about how to protect swine without harsh side effects from commercially available vaccines. Our lab proposed that capsular polysaccharide (CP), a molecule that is necessary for disease by related bacteria, is also produced by Hps, and can be used in a protective and safe subunit vaccine as well as in field diagnostic tests for rapid detection of the bacteria. To support this hypothesis, we outlined three objectives to explore the role and potential use of CP to protect against Hps.

We purified and characterized CP from several strains of Hps, and determined that each CP contained similar sugars, but in different amounts and structures. We also discovered that the production of CP is variable. In other words, environmental conditions control whether the CP is present on the cell or not. For instance, when the bacteria are grown on agar medium CP was not found, but when the bacteria were grown in liquid culture the CP was present. In addition, the presence of sodium bicarbonate, a molecule found in increased amounts in the bloodstream during episodes of stress (such as weaning, transport, and prior infection) enhanced CP production. Increased bicarbonate levels in swine before Hps infection may be an indicator the animals are more susceptible to this bacterial infection.

We generated antibodies against purified CP and used the antibodies to confirm that CP can induce an immune response, and is the serotype-specific antigen (there are 15 known serotypes). These antibodies also proved to be useful in research assays to further characterize the role of CP in protection against Hps. For example, we determined that bacteria that lack CP are able to be killed by normal host serum, but that bacteria expressing CP were resistant to normal serum unless CP antibodies were also present. We also hypothesized that a CP-protein complex could generate a robust antibody response to the CP in piglets, but the level of protection against Hps infection that these antibodies could provide is still being evaluated.

Finally, we incorporated antibodies to the CP into a rapid diagnostic test to determine if disease in swine is due to Hps, and to assist with proper typing of various Hps clinical isolates. The diagnostic test involved permanently binding CP antibodies to latex beads, which agglutinate, or clump together, in the presence of CP as the antibodies react to CP found on whole cells or is shed into the infection site. The beads agglutinate within a minute of mixing with a positive sample of the identical serotype, and significantly less or not at all with a different serotype. Furthermore, the antibody-sensitized beads agglutinated when mixed with lysed bacteria grown on agar medium, the current industry standard for the preparation of the bacteria for clinical serotyping, but not with unlysed bacteria. These results indicates that CP is present within the bacteria grown on agar medium, but appears not to be expressed on the cell surface unless triggered by the proper environmental stimuli.

Our results have identified the CP as the molecule responsible for Hps serotype specificity, as a virulence factor, and though still preliminary, as an immuno-protective antigen. The results of our work will lead to safer, more efficient and more rapid diagnostic assays and prevention of Hps infection in swine herds without the use of potentially toxic whole cell bacterins. While more research is necessary to finalize these products for commercial use, the identification of CP as a factor in Hps infection and diagnosis will positively influence the future directions of swine health research.

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Keywords
H. parasuis, swine health, capsular polysaccharide, bacterial diagnostics, subunit vaccine

Scientific Abstract

Background: *Haemophilus parasuis* (Hps) 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 and characterize the Hps capsular polysaccharide (CP), determine its role in serotype specificity and virulence, determine if CP can produce an immune response, and develop diagnostic and protective products to help prevent widespread Hps infection within a swine herd.
Materials and Methos: CPs were purified from Hps serotypes 4, 5, 9, and 13 using enzyme digestion, phenol extraction, and ultracentrifugation. The CP electrophoretic profiles were visualized by alcian blue/silver-staining and compared to CPs from other Pasteurellaceae. CP glycose composition was determined by gas chromatography-mass spectorrometry (GC-MS). Rabbits were immunized with CPs to generate antisera for immunofluorescence microscopy (IF), immunoassays, and bactericidal assays. CP was conjugated to the carrier protein Cholera Toxin B (CTB), and used to immunize piglets before challenge with Hps serotype 5 to determine the protective efficacy of the antibody response to Hps CP. CPs from serotypes 4, 5, and 9 were also conjugated to latex particles to create a diagnostic agglutination assay for detection and typing of various Hps serotypes.
Results: The electrophoretic profiles of CP from broth-grown Hps resembled those of other Pasteurellaceae, but CP was not isolated from Hps grown on agar medium. The presence of bicarbonate also enhanced production of CP. The composition of CP from the different serotypes examined by GC-MS was similar, but in different ratios and likely different in structure. The CP was visible on broth-grown Hps incubated with homologous CP antiserum by IF and immuno-transmission electron microscopy, but not on agar-grown cells or broth-grown heterologous Hps strains. Antiserum reacted strongly with homologous CPs of Hps grown in broth by enzyme-linked immunosorbent assay, but weaker cross-reactivity occurred with CPs from other Hps serotypes. Western blotting with homologous and heterologous antiserum confirmed that the lipopolysaccharide was highly conserved and not serotype-specific. Agar-grown Hps cells were highly susceptible to killing by normal swine serum, but broth-grown Hps were serum-resistant unless homologous anti-CP serum was present. The CP conjugate produced a robust antibody response to CP, as determined by immunoassays, but because the control animals also survived and contained some antibodies to Hps CP, the vaccine study remains inconclusive. Latex beads sensitized with antibodies against CPs agglutinated when mixed with purified CP or whole cells grown in broth. The latex beads were most reactive with homologous CP and whole cells, but also reacted weakly with heterologous cells. When agar-grown cells were lysed, the lysate produced an agglutination response to homologous sensitized latex particles, whereas unlysed agar-grown cells did not.
Conclusions: Unlike most CPs that are constitutively expressed, expression of the Hps CP was upregulated by environmental factors, such as growth in broth, and the addition of bicarbonate. While CP was identified as the immunodominant antigen, cross-reactivity did occur in highly sensitive assays. Because the Hps serotype-specific antigen is carbohydrate and was not lipopolysaccharide, these results indicate the CP only is serotype-specific. CP was required for bactericidal resistance to normal serum, but antibodies to the CP were bactericidal, indicating the CP protects the bacterium from host immunity, but that antibodies to the CP may be protective. The vaccination study produced inconclusive results, but due to the baseline antibody titers that the piglets possessed before challenge, there is a possibility that a previous infection with *E. coli* conferred some cross-protection, due to potential similarity in CP types between the two bacteria. The latex agglutination assay

demonstrates a solution to the problem of the slow and inconsistent diagnostic and typing standards that are currently available, but further calculations and adjustments to the assay are necessary to eliminate any cross-reactive agglutination. Thus, the determination that the Hps CP is an important virulence factor and is serotype-specific, and that expression of the CP is controlled by environmental factors will help direct the future research in disease prevention and serotype tracking in Hps infections.

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. Symptoms are generally observed in piglets 3 weeks to 4 months of age due to the stresses of weaning, relocation, or prior infection. Currently, infections are treated with a course of β-lactam antibiotics. Vaccines (bacterins or a live-attenuated strain) are available, but current vaccines do not protect against all serotypes, and the endotoxin present in whole cells can cause dangerous side effects. *H. parasuis* can destroy up to sixty percent of a herd within 24-72h. Therefore, a safer, cost-effective *H. parasuis* vaccine and a rapid screening test for detection of virulent serotypes is 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. The available vaccines are directed toward serotypes 4, 5, and 13. However, the molecular determinants responsible for serotype specificity are undefined, although these antigens are known to be heat-resistant and presumed to be carbohydrate. The most common bacterial antigens associated with type specificity that are heat-resistant are capsular polysaccharide (CP) and lipopolysaccharide (LPS), or in the case of *H. parasuis* lipooligosaccharide (LOS). The CP is the serotype-specific antigen in many genera of the Pasteurellaceae family, but the role of the *H. parasuis* CP has yet to be determined. The CP may also contribute to bacterial virulence by promoting resistance to phagocytosis and complement-mediated killing. As a result, antibodies to the CP may 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. We have confirmed that a CP is present on most *H. parasuis* isolates, but appears to be regulated because the CP could only be isolated from shaken, liquid cultures, and not from cultures grown on agar medium. Furthermore, we present preliminary evidence that the *H. parasuis* CP is the serotype-specific antigen and contributes to resistance to host defenses.

Objectives

a. Isolate, purify and characterize the CP from three serotypes of *H. parasuis*
b. Raise antibodies to the CP, use such antibodies to confirm that the CP is the serovar-specific antigen, and that antibodies to the CP are protective in mice.
c. Determine the protective efficacy of antibodies to the CP in pigs, and develop diagnostic tests to detect the CP.

Materials and Methods

Bacterial strains, media, and growth conditions: *H. parasuis* reference serotypes were acquired from Dr. Linda Zeller at the Iowa State School of Veterinary Medicine. *Actinobacillus pleuropneumoniae* strain J45 (encapsulated) and J45-C (a non-encapsulated mutant) [15] were used as controls. Bacteria were grown on chocolate agar plates (Remel, Lenexa) or PPLO-agar plates (BD, Franklin Lakes) supplemented with 100 µg/ml of nicotinamide adenine dinucleotide (NAD) (Acros, Geel) 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), and 1% glucose (Sigma, St. Louis) (PPLO+). Strains were grown on chocolate agar or PPLO+ or in 20 ml of PPLO+ broth. The cultures were shaken at 37°C to mid-log phase, determined spectrophotometrically using a Klett spectrophotometer and confirmed by viable plate count. This culture was used to inoculate 2 L of PPLO+ and grown to late mid-log phase (24 h) or stationery phase (72 h).

CP purification: CP was purified by modification of procedures previously developed in our laboratory for the *A. pleuropneumoniae* CP. The bacteria were scraped from agar plates into phosphate buffered saline, pH 7.2

(PBS), or grown on PPLO+ agar for about 48 h prior to inoculation into broth, which was allowed to grow to mid-log phase and 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). The pellets from both agar- and broth-grown cultures were resuspended in 50 ml of distilled water, heated at 121°C for 30 minutes, the mixture subjected to centrifugation, and the supernatant saved. 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 RNAse (Riboshredder, EpiCentre, Madison) and DNAse (Sigma) for 1 h each at 37°C, followed by Proteinase-K (Sigma) overnight at 56°C. The mixture was then extracted with 50% phenol (Fisher Scientific, Fair Lawn) for 30 min at room temperature and subjected to centrifugation. The aqueous phase was removed and distilled water was added to the phenol phase, and the extraction procedure was repeated until no proteinaceous material was present at the water-phenol interface. The aqueous phases were pooled and dialyzed against distilled water until there was no detectable phenol odor. Any LOS in the samples was removed by centrifugation 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 (39 x 1 cm column at a flow rate of 1 cm/10 min). Carbohydrate-positive fractions that eluted in the void volume were pooled, dialyzed, and lyophilized. Reducing carbohydrate was determined by a reduced volume phenol-sulfuric acid assay. Protein concentrations were determined by BCA assay (ThermoPierce, Fair Lawn).

LOS purification: LOS was extracted using hot, aqueous phenol. Bacteria were scraped from agar plates into reacti-vials (Pierce) containing 1 ml endotoxin-free water, and mixed with 50% 70°C phenol. The bacterial suspensions were mixed for 30 min at 70°C, then cooled on ice for 5 min before centrifugation at 10,000 x g for 15 min. The aqueous phase of each sample was saved, made 30 mM in sodium acetate and 5 volumes of cold 90% ethanol were added to each sample. The samples were incubated at -20°C overnight and were once again subjected to centrifugation at 10,000 x g for 15 min. The supernatant was decanted and the pellets were resuspended in 50 µl of HPLC water and stored at -20°C for electrophoresis and immunoblotting.

Gel electrophoresis: CP (20 µg) was applied to a 25% continuous acrylamide gel in sample buffer and electrophoresed. The CP electrophoretic profile was visualized by staining the gel with Alcian Blue and Silver stain (Bio-Rad, Hercules). LOS (2 µg) was electrophoresed in 15% discontinuous acrylamide gels containing urea in the separating gel, and stained with ammoniacal silver (Tsai and Frasch).

Antiserum: 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 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 saline 4 weeks thereafter, and then every two weeks until the titer reached >1:10,000 by enzyme-linked immunosorbent assay (ELISA). Hps whole cell antisera to serotypes 4, 5, and 9 were kindly provided by Drs. Susan Brockmeier and Crystal Loving at the National Animal Disease Center in Ames, Iowa.

Immunoblotting: Two µg of purified CP or 2 x 10^5 of *H. parasuis* were blotted onto nitrocellulose membrane (Whatman, Dassel) 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 a 1:500 dilution of either anti-CP serum (for CP and whole cells) or whole-cell anti-Hps serum (for LOS) in 5% NFDM in PBST was added. 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 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).

ELISA: Ten µg/ml of purified CP or 10^6 cells suspended in PBS supplemented with 2 mM $MgCl_2$ (PBS-M) were added to wells of Immulon 4HBXmicrotiter plates (Immulon, Fair Lawn, NJ). All incubations were carried out at 37°C for 1 h, and the plates washed at least 3 times with PBST. Nonspecific

binding was blocked with 2% NFDM in PBST, the plates were washed, and incubated with serial dilutions of antiserum in 2% NFDM in PBST. The plates were washed, and HRP-tagged anti-rabbit IgG (Jackson Labs, Bar Harbor) diluted 1:5000 in PBST was added. After thorough washing, TMB-peroxidase (ThermoPierce, Fair Lawn, NJ) was added until color developed. The reaction was stopped with 1 M H_2SO_4 and the A_{450} determined.

Bactericidal assay: Bacteria were grown on solid agar medium, or grown in broth to mid-log phase (10^9 CFU/ml, determined spectrophotometrically) or for 24 h. The cells were washed in PBS, and resuspended to 10^3 CFU/ml in PBS supplemented with 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (PCM, Sigma). Swine sera from non-immune animals, 20% precolostral calf serum (PCS) as a complement source, or 10-50% antiserum supplemented with PCS were added to the cells. Twenty µl of the mixture was immediately spread onto 2 quadrants of a chocolate agar plate (0), 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). The chocolate plates were incubated for 24 h, and percent viability was determined by dividing the number of colonies from mixtures incubated for 60 min by the number of colonies from mixtures at 0 min incubation, and multiplying by 100.

Transmission Electron Microscopy (TEM): Bacteria grown to mid-log phase in PPLO+ broth were resuspended to 10^4 CFU/ml, washed once with PCM, and then incubated with homologous or heterologous CP antisera for 1 h at 37°C, washed with PBST, and then fixed in cacodylate buffer supplemented with 5% glutaraldehyde and 0.15% ruthenium red (Sigma) for 2 h at room temperture. The bacteria were fixed in 4% agar, washed 5 times in cacodylate buffer, and post-fixed with 2% osmium tetroxide for 2 h. The agar blocks were washed as above, and the samples were dehydrated in 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 an electron microscope at an accelerating voltage of 80 kV.

Immunfluorescence microscopy: Bacteria grown on agar medium, or to mid-log phase or for 24 h in broth, were washed and resuspended in PCM. The bacteria were diluted to about 10^3 CFU/ml, spread onto 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 washed with PBST with rocking for 10 min, allowed to dry, and a 1:20 dilution of fluorescein isothiocyanate (FITC)-anti-rabbit or mouse IgG (heavy and light chain, Fisher, Fair Lawn NJ) was added. The slides were incubated in a sealed, humid chamber for 1 h at 37°C, placed in PBST 10 min, dried, then 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.

CP conjugate preparation: To optimize the host immune response to the CP, a carrier protein was conjugated to CP in a novel fashion, utilizing the high affinity bond between streptavidin and biotin. CP was conjugated to hydrazide-linked streptavidin (ThermoPierce, Fairlawn NJ), which was then attached to biotinylated cholera toxin B (Sigma) using the recommended manufacturers' instructions. Ten mg of CP was oxidized in 1 ml 0.1M sodium acetate buffer, pH 5.5, and then mixed 1:1 with a buffer of 20 mM sodium m-periodate 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 excels periodate. The CP was then added to dissolved hydrazide-streptavidin at 5 molar excess, and mixed end-over-end for 2 h at room temperature. The integrity of the CP-streptavidin intermediate was measured by sandwich ELISA by the protocol described above, using homologous antisera as a coating antigen and HRP-tagged biotin as the reporter conjugate. The CP-streptavidin intermediate was then added to the biotinylated cholera toxin B and allowed to mix at room temperature overnight. The final CP-cholera toxin B (CP-CTB) product was determined to be conjugated by electrophoretic analysis with Coomassie Blue staining and Alcian Blue/silver staining to visualize the conjugate The CP-CTB was lyophilized until immunization of pigs at the National Animal Disease Center, Ames, IA.

Protective efficacy of CP antibodies in piglets: Eight caesarian-derived piglets were split into two groups: a control group receiving intramuscular doses of sterile saline during each round of inoculation, and an immunization group receiving 100 ug of CP-CTB conjugate in sterile saline supplemented with TiterMax adjuvant (Norcross, GA). 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. Piglets were immunized on day 0, boosted on day 21, and challenged on day 54. All animals received a lethal dose of Hps 5 (Nagasaki strain), intratracheally. Animals were monitored daily for changes in health, and were humanely euthanized either when they became moribund or at day 62. Necropsy was performed on each of the piglets to determine the presence of Hps in various tissues. Antibody titers were determined by ELISA using CP from Hps serotype 5 and CP-CTB as antigen.

CP conjugation to latex particles: Hyperimmune rabbit serum against Hps 4, 5, or 9 was passed through an Protein A/G affinity purification column (AmiconCorp., Danvers, MA) to purify the IgG component. Typically, 15 to 20 mg of IgG was isolated per 5 ml of adsorbed immune serum, as determined by protein assay as described above. 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) 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 washes were done in 1.5 ml microcentrifuge tubes at 14,000 x *g* for 6 min at room temperature unless otherwise stated. Following the final wash, the particles were suspended 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 SLP were transferred to microcentrifuge tubes and washed three times with 0.01 M borate buffer, pH 8.0. The SLP were resuspended in 1.2 ml of borate buffer and transferred to clean 1.8-ml cryovials. Concentrations of IgG ranging from 200 to 2,400 mg/0.5 ml of particles were added to the SLP, which were then rotated end-over-end overnight at room temperature. To block nonspecific binding, 50 ml of 0.25 M ethanolamine was added to the SLP and rotation was continued for 30 min. The SLP were transferred to 1.5-ml microcentrifuge tubes and centrifuged for 10 min. The supernatant was saved for protein determination by BCA assay, and the SLP were resuspended in 1% bovine serum albumin in borate buffer and rotated for 30 min at room temperature. The SLP 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).

Latex agglutination assays: CP was resuspended in PBS at 1 mg/ml, and cells were resuspended at a concentration of 10^9 CFU/ml in PCM. Cells grown on agar medium were either scraped directly into PCM, washed once and resuspended, or were scraped into PCM, autoclaved for 90 min to lyse the cells, cooled, and the lysate and supernatant were separated by centrifugation using the whole cell method for CP purification. Each CP and cell preparation was diluted 1:10 serially in PCM for at least 5 dilutions. The latex agglutination test was performed on a glass slide. Sensitized latex particles (SLPs) were mixed with sample of CP or cell preparation at a ratio of 5 ul:15 ul. The slide suspensions were mixed manually using a pipet tip and then rotated slowly to ensure homogeneity of the sample. This was done until strands of agglutinated latex/antigen particles formed, or until 5 min had elapsed. An agglutination score was ascribed to each reaction: 4 for instantaneous agglutination upon mixing, 3 for agglutination before 1min, 2 for agglutination before 3 min, 1 for agglutination before 5 min, and 0 for no agglutination after 5 min.

Results

Objective A: Figures 1-5
Objective B: Figures 6-11
Objective C: Figures 12-17

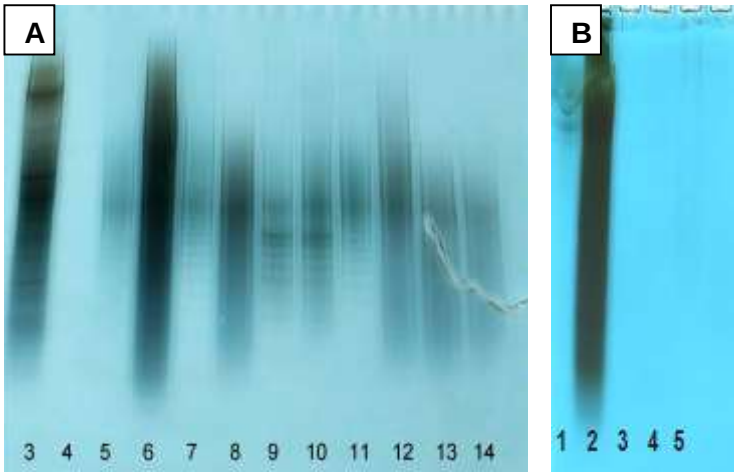


Fig. 1: Electrophoretic profiles of CP isolated from *A. pleuropneumoniae* (App) (control) and *H. parasuis* in broth (A) and agar (B). Lanes: (A) 3, CP from App; 4, blank; 5, 7, 9, 11, and 13, CP from *H. parasuis* serotypes 4, 5, 9, 13, and 14 isolated from broth supernatant, respectively; 6, 8, 10, 12, and 14, CP isolated from *H. parasuis* serotypes 4, 5, 9, 13, and 14 isolated from broth-grown cells, respectively. (B) 1, blank; 2, CP from App isolated from agar plates; 3-5, extracts from *H. parasuis* 4, 5, and 9 grown on agar, respectively.

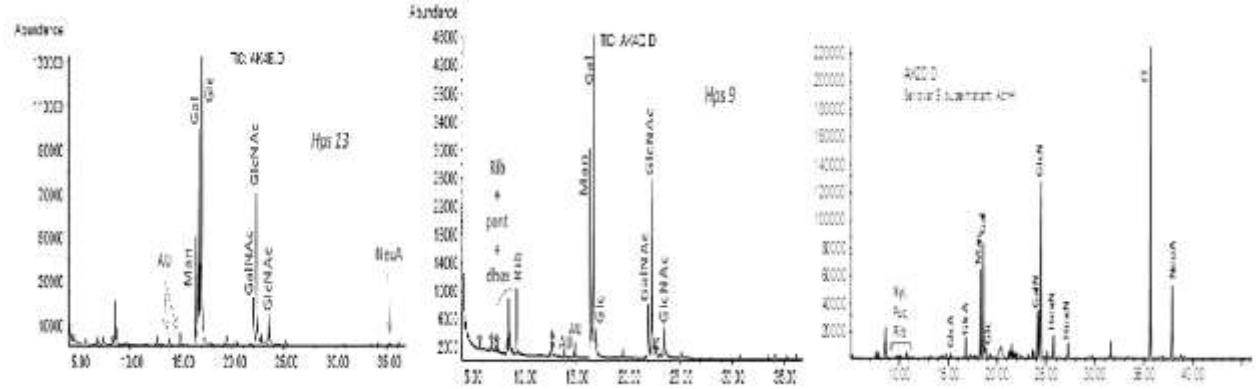


Fig. 2. GC-MS analysis of glycoses from *H. parasuis* serotypes 13, 9, and 5 CP (*H. parasuis* 4 CP not shown). Xyl (xylose), Fuc (fucose), and Rib (ribose) are likely contaminants. FT (internal standard). GlcA (glucuronic acid), Man (mannose), Gal (galactose), GalN (galactosamine), GlcN (glucosamine), NeuA (neuraminic [sialic] acid), HexN (hexosamine). Although similar glycoses are present, their concentrations vary considerably, and likely their linkages.

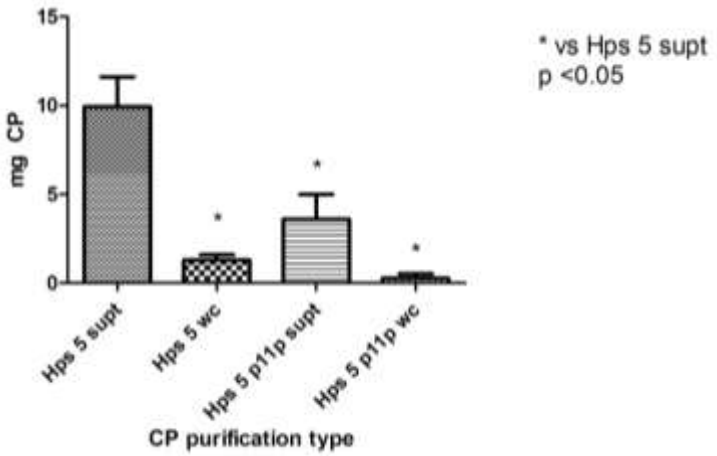


Fig. 3. Loss of CP production on solid media. Hps 5 bacteria were passed 11 times on PPLO$^+$ agar, and then grown in PPLO$^+$ broth for CP extraction to compare with the same strain that was not passed, but grown under the same broth conditions. Supt, CP present in the broth supernatant; wc, CP isolated from cells grown on agar; p11p supt, Hps 5 passed 11 times on PPLO$^+$ agar and then CP isolated from supernatant of cells grown in broth; p11 wc, Hps 5 passed 11 times on PPLO$^+$ agar and then CP isolated from cells grown on agar. The amount of CP present from cells passed in agar was significantly diminished ($p < 0.05$) compared to the parent strain.

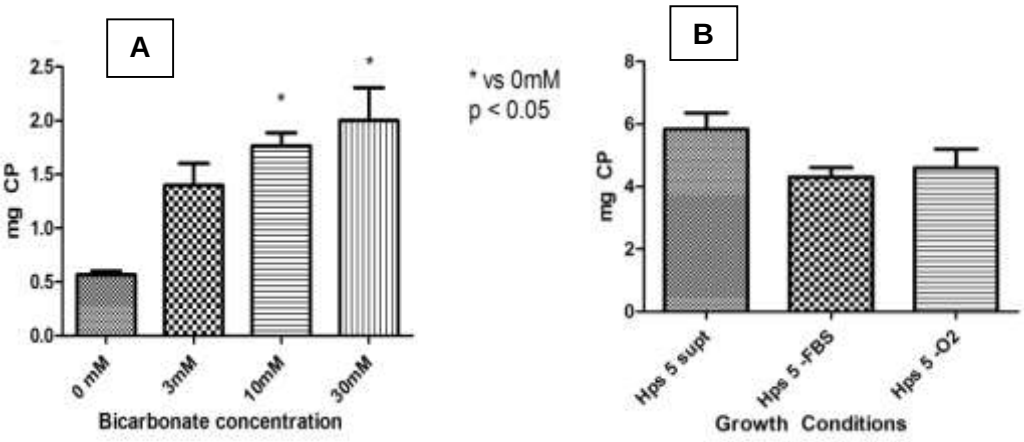


Fig. 4. Growth conditions affecting CP production. Hps cells grown in nutrient-starved media (A) or with the addition of bicarbonate (B). Supt- PPLO$^+$, -FBS- grown without FBS present, -O2- grown with Oxyrase. CP production was significantly increased with the addition of bicarbonate, but was still produced and did not significantly change with media absent of oxygen or FBS.

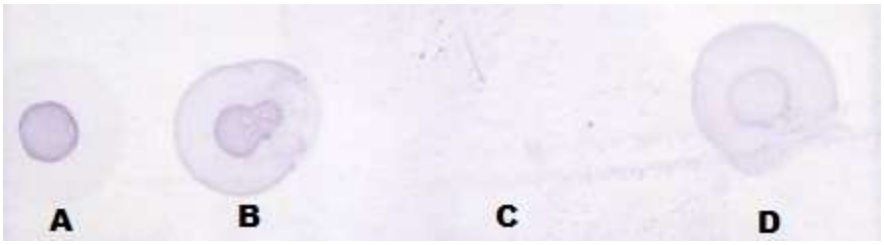


Fig 5. Dot blot of Hps 5 cells grown on agar with or without bicarbonate supplementation and reacted with antiserum to Hps serotype 5 CP. A, Hps 5 purified CP; B, broth-grown whole cells; C, agar-grown cells on

PPLO$^+$, D, agar-grown cells on PPLO$^+$ supplemented with 30 mM bicarbonate. The addition of bicarbonate to the agar medium enhances expression of CP on the cell surface.

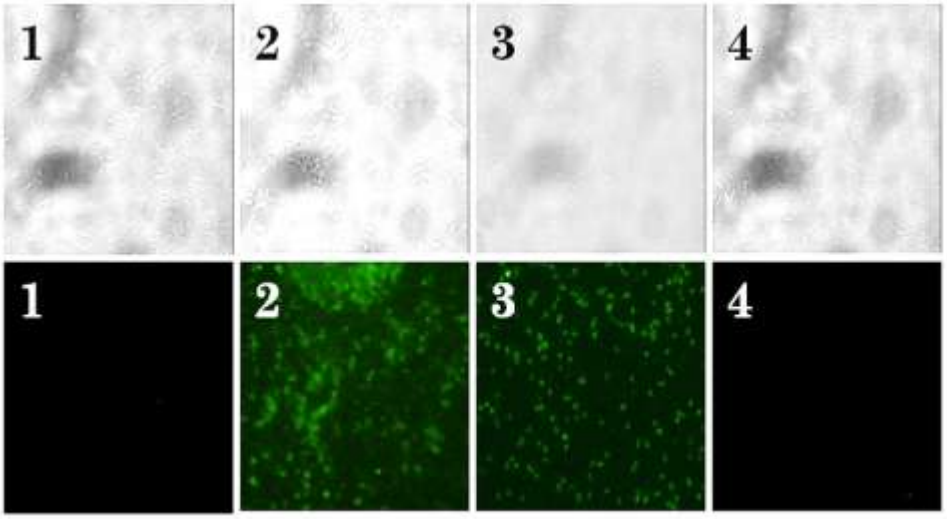


Fig. 6. Indirect fluorescence antibody labeling of CP on *H. parasuis*: Serotype 5 cells were grown on agar medium (1), in broth to mid-log phase (2), or in broth for 24 h (3 and 4), and were incubated with (1-3) or without (4) antiserum. FITC-labeled anti-rabbit IgG was added, mounted in 50% glycerol, and exposed to bright field (top) or FITC-filtered (bottom) lighting.

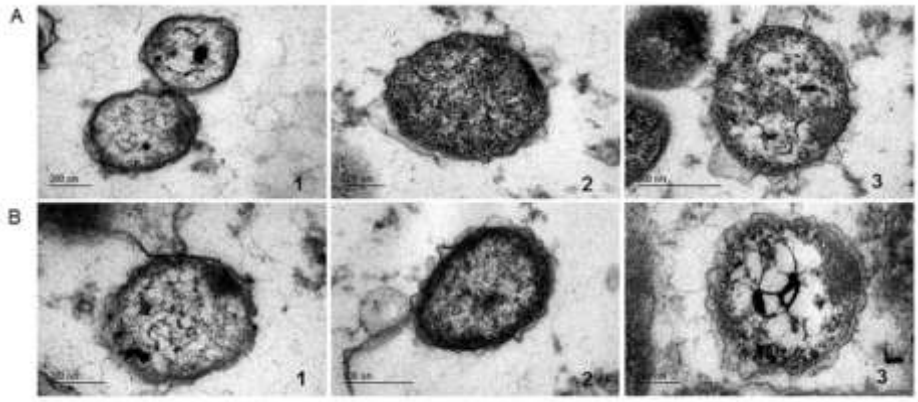


Fig. 7. Immuno-EM with rhuthinium red staining of *H. parasuis* cells grown on agar (1) or in broth (2, 3). Cells from serotypes 4 (A) and 5 (B) were grown on agar (1), or in broth to mid-log phase (2), or for 24h (3), washed, and incubated with homologous antibody to CP. Cells were then stained with 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).

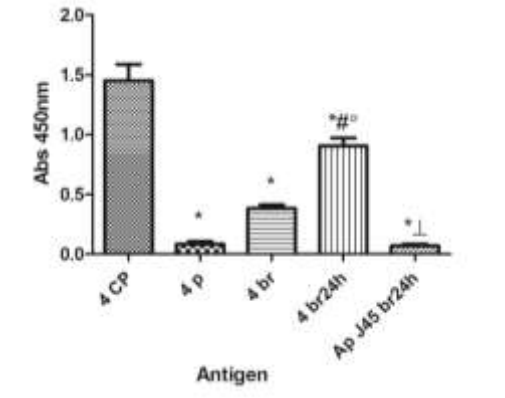


Fig. 8. Reactivity of CPs with homologous antiserum by ELISA. Antiserum to CP of serotype 4 was tested for reactivity to purified CPs and whole cells. CP, purified CP; p, cells grown on agar; br, cells grown in broth to mid-log phase; br24h, cells grown in broth for 24h; Ap J45 br24h, cells of *A. pleuropneumoniae* (control) grown in broth for 24 h. Similar reactivity occurred when CP and whole cells of serotypes 5, 9, and 13 were incubated with homologous antiserum (not shown).

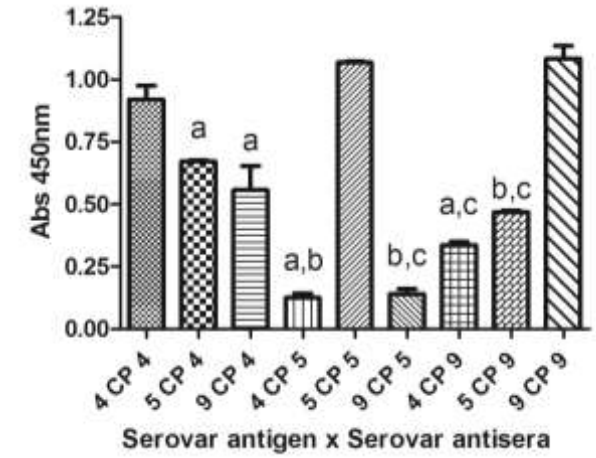


Fig. 9. Cross-reactivity of CPs with heterologous antisera by ELISA. Antisera to CP reacted strongly to homologous CP, but not significantly to heterologous CPs ($p < 0.05$). a, reactivity of antisera to CP 5 or 9 with CP 4, or reactivity of antisera to CP 4 with CP5 or 9 compared with reactivity of antiserum to CP 4 with CP 4; b, reactivity of antiserum to CP 5 with CP 9 or reactivity of antisera to CP4 or 9 with CP 5 compared to reactivity of antiserum to CP 5 with CP5; c, reactivity of antiserum to CP 9 with CP5, or reactivity of antisera to CP 4 or 5 with CP 9 compared to reactivity of antiserum to CP9 with CP 9.

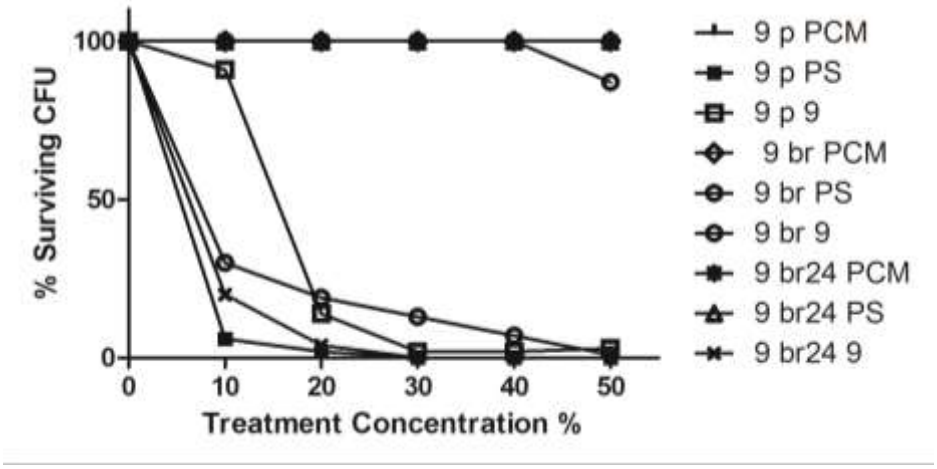
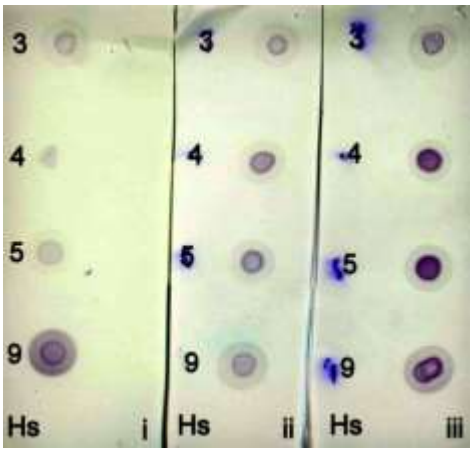


Fig. 10. Bactericidal Assay: *H. parasuis* cells were grown on agar (p) or in broth to mid-log phase (br), or for 24 h in broth (br24). The bacteria were washed, diluted, and resuspended in PCM containing no supplements (PCM), non-immune swine serum (PS), or homologous antiserum (#) supplemented with pre-colostral calf serum. The cultures were spread on agar plates at 0 min or after 60 min incubation at 37°C. After 24 h incubation colonies were counted and percent survival was determined. There was no loss of viability in cultures incubated in PCM only, but agar-grown cells were sensitive to killing by fresh, non-immune swine serum. Bacteria grown in broth were resistant to normal serum unless supplemented with homologous antiserum. Results are representative of results obtained with serotypes 4 and 5 (data not shown).

A



B

Fig. 11. LOS electrophoretic profiles and antigenic reactivity. Hps 3, 4, 5, and 9 was grown on agar medium prior to LOS microextraction. A, gel electrophoresis with silver-staining; B, antisera to whole cells of Hps serotypes incubated with LOS of serotypes 4 (i), 5(ii), and 9 (iii). *Histophilus somni* 2336 cells (Hs) were used as a control. The LOS from each of the serotypes had little to no heterogeneity in electrophoretic profile, and all

serotypes were reactive with whole cell antisera to each serotype, indicating that LOS is not the serotype-specific antigenic determinant.

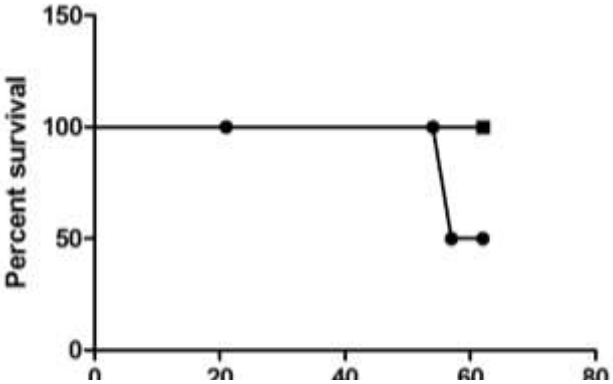


Fig 12. Survivial of piglets following immunization with CP-CTB and challenge with Hps 5. Piglets were divided into two groups, one group received PCM only (●, control), and one group received the CP-CTB conjugate (■, CP-CTB). Pigs were immunized on day 0, boosted on day 21, and challenged with a lethal dose of Hps 5 Nagasaki strain on day 54. Only one piglet from the control group succumbed to infection, whereas the rest of the piglets survived the duration of the study.

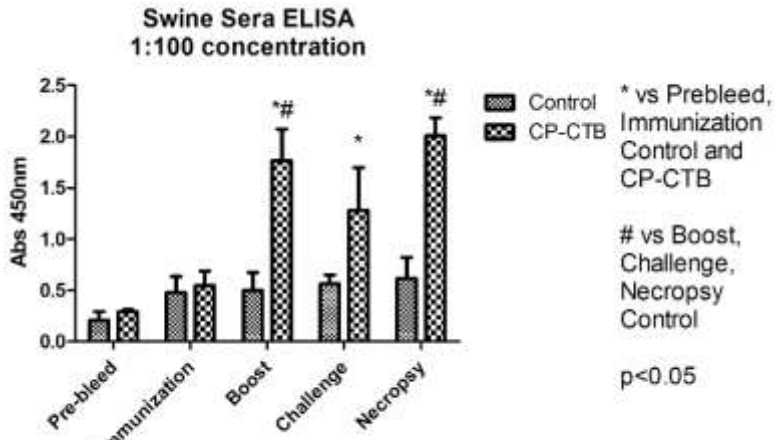


Fig. 13. Reactivity of sera from immunized pigs to purified CP. Each piglet in the study was bled initially for baseline CP antibody titers, and then before immunization, when boosted, at challenge, and when euthanized. Each serum sample was tested for reactivity to purified Hps 5 CP by ELISA, as described above. All pigs had a positive titer to CP, but the pigs immunized with CP-CTB had a significantly greater response ($p < 0.05$) than the controls.

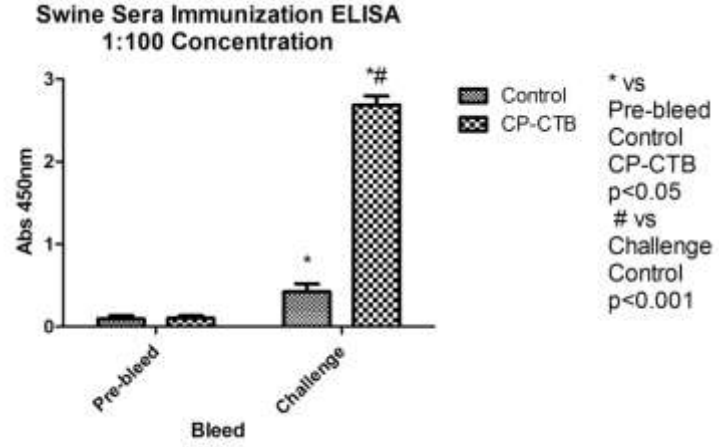


Fig. 14. Reactivity of control and immunized pigs to the CP-CTB conjugate. Piglets were bled and immunized as described above. Serum samples obtained prior to the initial immunization and at challenge were tested by ELISA to determine the antibody response to the CP-CTB conjugate. Although the control 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 ($p < 0.01$).

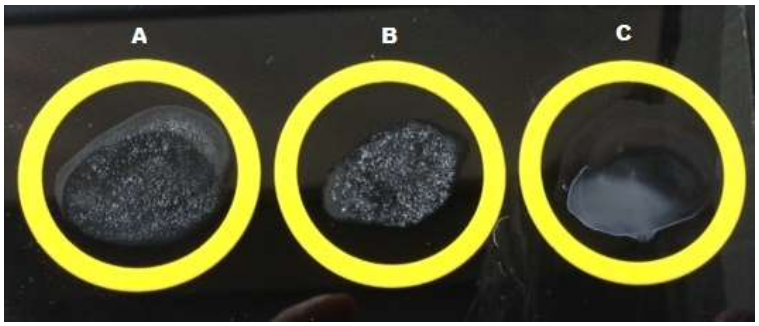
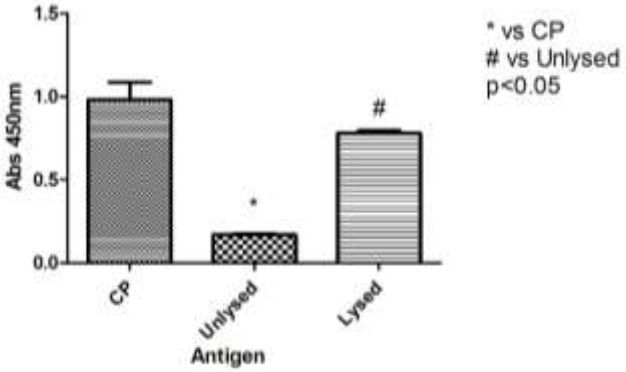


Fig. 15. Latex agglutination of purified CP and CP from broth-grown and lysed agar-grown cells. Antibody-sensitized latex particles were prepared as described in methods, and CP and cells were prepared as previously described. Five ul of latex particles were mixed with 15 ul of purified CP (A), CP from broth-grown cells or agar-grown lysed cells (B, representative of all results), or agar-grown unlysed cells (C). Latex agglutination occurred almost instantaneously with high concentrations of purified CP or cells, whereas unlysed cells did not agglutinate at all after 5 min of mixing.

Fig. 16. Quantification of CP from lysed Hps cells. Cells were grown on agar medium as described above, and were either lysed by 90 min of autoclaving, or scraped directly into PCM. Cells and lysates were subjected to centrifugation at 8,000 x *g*, and the supernatant was collected, the CP purification protocol applied, and the product resuspended and quantified by ELISA. Lysed cells had a comparable amount of CP to the product from broth-grown cells, but unlysed cells had significantly less reactivity to homologous CP antiserum.



Discussion

Hps serotypes produce CPs that are the predominant serotype-specific antigenic determinant and contribute to virulence, as shown by immunoassays and carbohydrate analysis. The amount of CP produced and expressed on the cell surface is dependent on environmental factors, such as broth or agar, and the presence of bicarbonate. Growth in broth may mimick physiologic systemic conditions during dissemination. The current understanding of Hps infection 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. The surface expression of CP would be required to protect Hps in the bloodstream and tissues by evading opsonization and bactericidal factors. The presence of sialic acid on the CP also contributes to evading detection by the immune system, as sialic acid is a normal component of host tissues, and is recognized as “self”. The presence of bicarbonate enhances the amount of CP produced and expressed on Hps cell surface, which would further enhance virulence of the bacterium. Bicarbonate is a source of carbon dioxide, which is required for Hps growth, but is also a hallmark molecule secreted during times of physiologic stress to manage host pH. Stressful conditions that exist for piglets include weaning, transport, and recovery from previous infection. Bicarbonate as a reporter molecule for bacteria interacts with adenylate cyclase tmAC cell receptors, causing a kinase activation cascade within the cell and potentially creating downstream phosphorylated products that can regulate gene expression, possibly for such loci at the CP production and transport locus. Further exploration of the regulation of CP would be beneficial to understand this virulence mechanism, as well as understanding that eliminating sources of stress will lessen susceptibility of swine to Hps infection.

The discovery of CP as the antigenic determinant and virulence factor of Hps is important, as little was previously known regarding the role of CP in virulence and type specificity. Many genera of the Pasteurellaceae family produce CPs, and in many cases antibodies against CPs from non-toxin producing bacteria are protective against infection. The identification and characterization of CP can lead to potentially safer treatments and preventative measures for production swine. While CP produces an antibody response in piglets when immunized with a carrier protein conjugated to CP, it remains inconclusive as to whether the current level of antibody response produced against a CP conjugate is adequate to confer protection against

Hps infection. Several mitigating factors could potentially explain these inconclusive results. The unexpected survival of control animals after a lethal dose of Hps was administered could be due to the caesarian-derived piglets becoming infected with an antibiotic-resistant strain of *E. coli*. As a result, 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 in vivo antibody response to CP and potential protection against a lethal challenge dose of Hps. While the CP-CTB immunized animals 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 in three of the control animals increased by the end of the challenge. The possibility of cross-reactive antibodies produced against an *E. coli* antigen is a potential explanation. The CP locus of Hps has strong homology to the type I CP locus of *E. coli*, but it is still unknown if the similar sugars or CP structure existed in the strain of *E. coli* that affected the facility. We are currently waiting for further information about the pigs in the study from the NADC, and will proceed with the newfound knowledge from those reports to reconfigure the conjugate and conduct another trial.

When CP is conjugated to latex particles, it can be used as a rapid agglutination test for detection and serotyping of Hps. The success of the latex agglutination reaction with CP on whole cells demonstrates the possibility of developing a rapid diagnostic test for veterinarians considering Hps as an infectious agent in a production facility. Swine with Hps infections do not generally present with a single hallmark symptom, either clinically or during necropsy, and empirical treatment is not possible due to the breadth of potential causes of the symptoms or pathologies. The current method of diagnosis involves sending samples to a clinical laboratory, growth on agar medium, and/or PCR confirmation. Culture results require 48 to 72 hours and PCR often has to be sent out to a lab capable of performing the test and may take several days for a result. By the time a conventional diagnosis can be confirmed, a large percentage of the swine herd could be dead from rampant spread of disease. Currently, our threshold for homologous CP detection is 10 pg of purified CP, and 1.5×10^5 CFU of whole cells, which could easily be detected from biopsy or necropsy samples. While the same sugars are present in many of the Hps serotypes, they differ in amount and probably in linkage. Currently, the latex agglutination assay is being optimized for sensitivity and specificity and further testing will indicate it's potential for rapid diagnosis, as well as for serotyping. In determining conditions for agglutination, we lysed cells grown on agar medium, which is currently the standard protocol for serotyping clinical isolates. Current serotyping standards fall short of complete accuracy, with many strain being reported as either untypeable or multiple types, which is likely due to regulation of the capsule and cross-reactivity of CPs in some assays. Although cross-reactivity in typing assays is problematic, antigens in subunit vaccines that are cross-reactive would be advantageous in that some protection may be provided against strains of heterologous serotypes. A positive agglutination test using cell lysate shows that cells grown on agar are capable of producing CP, but the CP is not transported to the cell surface. Further molecular analysis is being conducted to determine the regulatory mechanisms responsible for CP production and transport.

Our work using the funding provided by the National Pork Board has allowed us to provide the following benefits to pork producers nationwide:

- Knowledge of the existence and regulation of the expression of the capsular polysaccharide of *H. parasuis*, and its role as the antigenic determinant and a virulence factor for Hps infection. This information provides a clear path for future research to better understand the bacterium.
- A potential subunit vaccine candidate. The current conjugate requires refinement. However, antibodies that have proven to mediate bacteria killing *in vitro* are being produced *in vivo*, which is optimistic for the future use of a safer, cost-effective subunit vaccine.
- A potential rapid diagnostic assay to detect Hps infection on-site at a potentially compromised pork production facility. With fine tuning for sensitivity and specificity, the assay will enable veterinarians to identify if Hps is the cause of infection and administer immediate treatment.

The National Pork Board was credited in the following publications and presentations:

- 2012, 2013, 2014 Annual Research Symposium, Virginia-Maryland Regional College of Veterinary Medicine. Blacksburg, VA
- 2012 Conference on the Pathogenesis of Bacterial Diseases of Animals. Prato, Italy
- 2013 Mid-Atlantic Microbial Pathogenesis Meeting. Wintergreen, VA
- 2014 Graduate Research Symposium, Virginia Tech. Blacksburg, VA (*note: award for best oral presentation by graduate student Anne CM Hyman)
- 2014 General Meeting of the American Society of Microbiology. Boston, MA
- 2014 Conference on the Pathogenesis of Bacterial Diseases of Animals. Prato, Italy
- Anne C. Michalenka Hyman, Thomas J. Inzana. Expression and type-specificity of the capsular polysaccharide of *Haemophilus parasuis.* In preparation.