Generation and evaluation of a *Glaesserella (Haemophilus) parasuis* capsular mutant

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Running Title: Evaluation of a *Glaesserella parasuis* capsule mutant

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Abstract

Glaesserella (Haemophilus) parasuis is a commensal of the upper respiratory tract in pigs and also the causative agent of Glässer's disease, which causes significant morbidity and mortality in pigs worldwide. Isolates are characterized into 15 serovars by their capsular polysaccharide, which has shown a correlation to isolate pathogenicity. To investigate the role capsule plays in *G. parasuis* virulence and host interaction, a capsule mutant of the serovar 5 strain HS069 was generated (HS069Δcap) through allelic exchange following natural transformation. HS069Δcap was unable to cause signs of systemic disease during a pig challenge study and had increased sensitivity to complement killing and phagocytosis by alveolar macrophages. When compared to the parent strain, HS069Δcap produced more robust biofilm and adhered equivalently to 3D4/31 cells; however, it was unable to persistently colonize the nasal cavity of inoculated pigs, with all pigs clearing HS069Δcap by 5 days post-challenge. Our results indicate the importance of capsular polysaccharide to *G. parasuis* virulence as well as nasal colonization in pigs.

Introduction

Glaesserella (Haemophilus) parasuis is the etiologic agent of Glässer's disease in pigs, which presents as a fibrinous polyserositis, arthritis and meningitis (1, 2). In addition, it can be a bacterial contributor to swine respiratory disease and is found as a commensal in the nasal cavity of healthy swine (1). *G. parasuis* isolates are classified into 15 serovars, based on gene content and diversity at their capsular polysaccharide loci and via the Kielstein-Rapp-Gabrielson typing scheme (3, 4). While it appears some serovars are more pathogenic and widespread, serotyping has shown an incomplete correlation with isolate virulence (3, 5, 6).

The importance of capsular polysaccharide as a virulence factor for encapsulated bacteria has been investigated (7-10), which indicates the capsule-deficient counterparts to encapsulated bacteria are much less likely to cause invasive disease and are often reduced to avirulence. The reduction in virulence seen in capsular mutants has been attributed to the role that capsule plays in adherence to host tissues and evasion of the host immune response, specifically inhibition of complement mediated killing and phagocytosis (11). Additionally, there is evidence indicating capsular polysaccharide is essential for some encapsulated bacteria to colonize the mucous membranes of hosts (12).

The functions of capsule have been partially evaluated in *G. parasuis* SH0165, a virulent serovar 5 strain (10). The capsular mutant (SH0165ΔcapD) was significantly less virulent than the wild type bacteria and unable to cause invasive disease in challenged pigs (10). SH0165Δcap was also highly susceptible to complement mediated killing as compared to the wild type bacteria (10). This presented evidence for the importance of capsule in causing invasive disease; however, the characteristics of the capsular mutant were not fully elucidated and the capacity of SH0165Δcap to colonize the swine nasal cavity was not evaluated. In this study, a capsule mutant of *G. parasuis* HS069 (HS069Δcap), a virulent serovar 5 strain, was used to examine the *in vitro* and *in vivo* characteristics of capsule. We evaluated HS069Δcap for susceptibility to complement killing, biofilm formation, attachment to porcine macrophage 3D4/31 cells, and phagocytosis by porcine alveolar macrophages. In addition, swine were challenged with HS069 and HS069Δcap to evaluate virulence, capacity for nasal colonization, and stimulation of host immunity.

Materials and Methods

Bacterial isolates

The virulent serovar 5 *G. parasuis* strain HS069 was isolated from the lung of a pig with clinical signs of respiratory disease (13). Production and verification of HS069Δcap is described within the following mutant generation and results sections. *G. parasuis* strains were grown on brain heart infusion (BHI) plates or BHI broth (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 0.1 mg/mL nicotinamide adenine dinucleotide (NAD+) and 10% horse serum (referred to as BHI+). *G. parasuis* strains were also grown on chocolate agar, made with Columbia Blood Agar Base (Thermo Fisher Scientific Inc., Waltham, MA) supplemented 7% defibrinated horse blood (TCS Bioscience Ltd., Botolph Claydon, England) lysed at 80°C for 10 minutes and 25 µg/mL NAD+. Media were supplemented with chloramphenicol (1 µg/mL) for selection purposes. All strains were grown at 37ºC with 5% CO_2.

Escherichia coli strain TOP10 (Invitrogen, Carlsbad, CA) was used as the cloning host. *E. coli* was grown in Luria Bertani (LB) broth or agar (Oxoid). Media were supplemented with ampicillin (100 µg/mL) or chloramphenicol (50 µg/mL) where required for selection.

Mutant construction

DNA manipulation: Genomic DNA extractions were performed using the DNeasy Kit (Qiagen, Hilden, Germany), plasmid DNA extractions were performed using a plasmid miniprep kit (Qiagen), and PCRs were performed according to the manufacturers' protocols with Phusion High-Fidelity DNA Polymerase mix (New England Biolabs, Ipswich, MA) for cloning and GoTaq green PCR mix (Promega, Madison, WI) for verification. PCR fragments purified by QIAquick PCR Purification Kit (Qiagen). Restriction enzymes were obtained from New England

Biolabs. Plasmid DNA was extracted from the agarose gel using Gel Extraction Kit (Qiagen). DNA concentrations were measured using a NanoDrop (Bio-Rad Laboratories, Hercules, CA).

Construction of plasmid pGEMT-neuAup-Cm-wzsdn: Plasmid pGEMT-neuAup-cat-wzsdn was constructed for deletion of the whole capsule gene cluster using a three-step cloning strategy. First, genomic DNA of *G. parasuis* isolate HS069 was used as a template to amplify the upstream and downstream regions flanking the 14-kb capsule locus (14, 15). The 647-bp upstream region of the *neuA* gene (neuAup) was amplified using primers P1 and P2 and the 731-bp downstream region of the *wzs* gene (wzsdn) using primers P3 and P4 (Table 1). In parallel, the chloramphenicol resistance cassette (*Cm*), containing the 9-bp DNA uptake signal sequence (USS) of 5'-ACCGCTTGT (16) was amplified from 50 ng linearized plasmid pUSScat (17) as the template with primers P5 and P6 (Table 1). Second, PCR products were digested and ligated into the pGEM-T vector. The PCR product of the upstream region of the neuA gene was digested with SacI /BamHI and the downstream region of the wzs gene was digested with SalI/ BamHI. The pGEM-T vector was digested with SacI /SalI. Restricted products were gel purified and ligated. The constructed plasmid was transformed into *E. coli* TOP10 and transformants were confirmed using PCR. After purification, the plasmid pGEMT-neuAup-wzsdn was verified by Sanger sequencing. Finally, the purified plasmid pGEMT-neuAup-wzsdn and PCR product of the Cm cassette were subject to BamHI restriction. The gel purified fragments were mixed, ligated and transformed into *E. coli* TOP10. The resulting plasmid pGEMT-neuAup-cat-wzsdn was extracted and confirmed by Sanger sequencing.

Construction of the whole capsule locus deletion mutant Δcap::Cm mutant of G. parasuis HS069: The plasmid pGEMT-neuAup-cat-wzsdn was linearized with SacI and used to transform *G. parasuis* HS069 using natural transformation method as described previously with some modifications (18). Briefly, HS069 was grown on chocolate agar overnight and suspended in BHI broth to achieve an $OD_{660} = 2$. Then, a 20 µl aliquot of a 1/10 dilution of the suspension was spread in a 10 mm area on prewarmed chocolate agar and 20 µl of 8 mM cAMP and 10 µl of donor DNA in TE buffer were added and mixed with the bacterial cells. The mixture was incubated at 37ºC overnight. Bacterial cells were scraped up, suspended in 300 µl BHI broth, and plated onto chocolate agar with 1 ug/mL chloramphenicol. After incubation at 37ºC for 2 days, suspected transformants were verified using PCR. For negative control, 10 µl of TE buffer without donor DNA was added to a bacterial spot. The deletion was also confirmed by whole genome sequencing using the Illumina HiSeq 250 platform and PacBio RS II Resequencing protocol of the SMRT Analysis software v.2.3.0. Assembly of the PacBio generated HS069 wild type was done using HGAP (19), circularized using Circlator v.1.1.3 (20), and polished using Quiver v.1. The Illumina reads were subsequently mapped onto the PacBio assembly to correct small indels. Comparison of HS069Δcap against the wild type was made by mapping the Illumina and PacBio reads of HS069Δcap against the finished HS069 wild type assembly.

Transmission electron microscopy (TEM) for capsule visualization

Capsule was visualized via transmission electron microscopy (TEM) using previously described methods (21-23). Briefly, *G. parasuis* grown on BHI+ plates overnight were suspended in 0.1 M cacodylate buffer with 2.5% glutaraldehyde and 0.1% ruthenium red and incubated for 2 hours at room temperature. Bacteria were pelleted and resuspended in 0.1 M cacodylate buffer with 2.5% glutaraldehyde and 1.0 mg/mL of polycationic ferritin and incubated for 30 minutes at room temperature. Bacteria were washed three times in 0.1 M cacodylate buffer. After staining with ruthenium red and ferritin, samples were post-fixed with 2% osmium tetroxide and rinsed three times in 0.1 M cacodylate buffer. The samples were processed through graded ethanols, propylene oxide, and embedded in Eponate 12 (Ted Pella Inc., Redding, CA). Following a 48 hour polymerization, thin sections were taken and stained with 4% uranyl acetate and Reynolds' lead stain. Sections were examined with a FEI Tecnai G^2 BioTWIN electron microscope (FEI Co., Hillsboro, OR).

Growth kinetics

Growth kinetics of *G. parasuis* HS069 and HS069Δcap were evaluated using a GeneQuant Pro spectrophotometer (Amersham PLC, Little Chalfont, United Kingdom). *G. parasuis* isolates were inoculated from a liquid overnight culture into BHI+ broth at an OD_{600} of 0.05. The cultures were incubated at 37ºC with 5% CO_2 and shaking at 200 RPM. OD_{600} readings were taken hourly for 8 hours.

Biofilm assay using microtiter plate

The microtiter plate assay for biofilm formation was adapted from a protocol described by Cassat et al. (24). Overnight cultures of *G. parasuis* were adjusted to an initial OD_{600} of 0.05, 0.125, or 0.25 in BHI+ broth. Diluted *G. parasuis* was plated in triplicate on a Nunc 96-well flat bottom plate (Thermo Fisher Scientific Inc., Waltham, MA) and incubated statically for 48 hours at 37ºC with 5% CO_2. Cultures were gently removed from the plate and washed three times with sterile PBS. Biofilm was fixed with 100% ethanol, allowed to dry for 10 minutes, and stained with 0.1% crystal violet. After 15 minutes at room temperature, plates were washed gently three times with PBS and allowed to dry overnight. Crystal violet was eluted from the biofilm with 150 uL of 100% ethanol for 10 minutes. The elution (120 uL) was transferred to a new 96 well plate and absorbance measured at 538 nm with a SpectraMax M5 (Molecular Devices, LLC, Sunnyvale, CA). Three independent replicates were performed.

Complement mediated killing

To assess sensitivity to complement mediated killing, *G. parasuis* cultures were treated with guinea pig serum (GPS) (Quidel, San Diego, CA). Heat inactivated GPS (30 minutes at 56ºC) was used as a negative control. *G. parasuis* cultures were grown on BHI+ plates and suspended in PBS to reach an OD_{600} of 0.42 ($1x10^8$ bacteria/mL). In a 96-well plate, 90 uL of GPS was added to 10 uL of *G. parasuis* (approximately 10^6 CFU). The *G. parasuis* and GPS incubated for 1 hour at 37ºC, 5% CO_2, 100 rpm shaking. Serial dilutions were plated on BHI+ plates.

Adherence capacity to porcine alveolar macrophage cell line (3D4/31 cells)

The interaction of *G. parasuis* HS069 and HS069Δcap with porcine alveolar macrophages was tested *in vitro* using the 3D4/31 cell line (ATCC, Manassas, VA). 3D4/31 cells were maintained in complete Advanced RPMI 1640 (Thermo Fisher Scientific Inc., Waltham, MA) as per ATCC's recommendations. For the assay, 3D4/31 cells were plated into 4-well chamber slides (ibidi USA Inc., Madison, WI) at $5x10^5$ cells/mL and allowed to adhere overnight. *G. parasuis* HS069 and HS069Δcap were added to the chambers to obtain an MOI of 10:1 and incubated for 1 hour at 37ºC and 5% CO_2. After incubating, 3D4/31 cells were washed to remove non-adherent bacteria and chamber slides were placed on ice. *G. parasuis* cells were incubated with mouse monocolonal antibody to the outer membrane protein P5 (provided by M. Gottschalk) for 30 minutes at 4ºC followed by incubation with goat anti-mouse IgG3 (SouthernBiotech, Birmingham, AL) for 30 minutes at 4ºC. Cells were fixed with ice cold 50:50 methanol:acetone for 10 minutes and dried. Images were taken using Nikon AR1+Si confocal microscope and evaluated using the NIS Elements software (Nikon Instruments Inc., Melville, NY). Bacterial intensity was evaluated using 10 random views.

Phagocytosis assessment using primary porcine alveolar macrophages

Porcine alveolar macrophages (PAMs) were isolated as previously described (25, 26), with modifications. Briefly, the lungs of healthy pigs were flushed with PBS repeatedly until around 250 mL of fluid was collected. The lavage fluid was centrifuged at 1000 RPM for 10 minutes. The pellet was washed twice and resuspended in complete RPMI-1640 medium (10% fetal bovine serum, 1 µg/mL fungizone, 100 U/mL penicillin, and 100 µg/mL streptomycin). PAMs were allowed to adhere to petri dishes for 2 hours at 37º C with 5% CO_2. After 2 hours, media and non-adherent cells were aspirated and adherent cells were washed with complete RPMI. Adherent cells were removed via cell scraping, washed twice with PBS, and resuspended in RPMI without antibiotics. Cells were counted and scored for viability using the Countess II Automated Cell Counter (Invitrogen, Carlsbad, CA). PAMs were plated into 48-well plates with $5x10^5$ PAMs per well and allowed to adhere for 20 minutes prior to starting the assay.

Bacterial stocks were generated by suspending agar grown HS069 and HS069Δcap in PBS with 50% glycerol at an OD_{600} of 0.42. The stocks were quantified via serial dilution and frozen at -80º C until use. HS069 or HS069Δcap stocks were diluted to obtain an MOI of 10:1 in 250 µL total volume per well (approximately $5x10^6$ CFU/well).

To assess phagocytosis, the media was aspirated and RPMI containing *G. parasuis* was added to each well. The PAMs were incubated with the bacteria for 1 or 2 hours and the supernatant was used to quantify non-phagocytosed bacteria. PAMs were isolated from four different animals and 2-4 replicates were completed per animal for each isolate. The CFU/mL was quantified for HS069 and HS069Δcap inocula. The log fold reduction was calculated by subtracting the remaining bacteria at hour 1 or 2 from the initial inoculum.

G. parasuis challenge

All experiments were approved by the National Animal Disease Center's Institutional Animal Care and Use Committee. Caesarian-derived, colostrum-deprived (CDCD) pigs were derived at the National Animal Disease Center. At 4 weeks of age, pigs were intranasally challenged with 2 mL (1 mL per nostril) of 1x10^8 CFU/mL *G. parasuis* HS069 (4 pigs) or HS069Δcap (5 pigs) suspended in PBS. Pigs were monitored for clinical signs (lameness, respiratory distress, lethargy, neurologic signs) and humanely euthanized when systemic signs of disease were noted. At necropsy, gross lesions were recorded and serum, nasal swabs, serosal swabs, joint fluid, lung lavage, and cerebral spinal fluid samples were obtained and plated for CFU counts. Serum and nasal swab samples were taken on day 0 and day 21 post-challenge. To investigate the vaccine potential of HS069Δcap, the pigs surviving challenge with HS069Δcap (5 pigs) were intranasally challenged with 2 mL (1 mL per nostril) of 1x10^8 of *G. parasuis* HS069 wild type on day 21 post-challenge with HS069Δcap. Pigs were monitored and treated as described above

A follow up study was conducted to evaluate nasal colonization of HS069Δcap in CDCD pigs. At 6 weeks of age, 6 pigs were inoculated intranasally with 2 mL (1 mL per nostril) of 1x10^8 CFU/mL suspension of *G. parasuis* HS069Δcap in PBS. Nasal swabs were obtained on day 0, 1, 3, 5, 7, and 14 post-challenge for *G. parasuis* detection. *G. parasuis* species specific PCR was run on these samples to detect bacterial colonization utilizing the primer set described by Howell et al. (27). Briefly, DNA was extracted from 50 µL of nasal swab samples using the MagMAX Pathogen RNA/DNA Kit (Thermo Fisher Scientific Inc., Waltham, MA). Extracted DNA was screened as previously described using *G. parasuis* species specific primers: HPS_219690793-F (5'-ACAACCTGCAAGTACTTATCGGGAT-3') and HPS_219690793-R (5'-TAGCCTCCTGTCTGATATTCCCACG-3') (27).

ELISA for serum antibody titer

Nunc MaxiSorp plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with 0.5 mg/mL of HS069Δcap sonicate in 100 mM carbonate-bicarbonate buffer (pH 9.6) at 4ºC overnight. Plates were washed with 0.05% Tween 20 in PBS (PBST) followed by blocking for 1 hour at 37ºC with 2% bovine serum albumin (BSA) in PBST. After washing with PBST, serum samples were serially diluted in 1% BSA/PBST and applied to wells for 2 hours at 37ºC. Plates were washed with PBST and HRP-conjugated secondary goat anti-swine IgG antibody (SeraCare Life Sciences, Milford, MA) was diluted 1:50,000 in 1% BSA/PBST added and incubated for 1 hour at 37ºC. ELISAs were developed using tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific Inc., Waltham, MA). TMB was added to each well, incubated at room temperature for 15 minutes, and the reaction halted with sulfuric acid (2 N). Absorbance 450 nm was measured on a SpectraMax M5 (Molecular Devices, LLC, Sunnyvale, CA).

Statistical analysis

Statistical analysis was completed using GraphPad Prism 7.03 (GraphPad Software, La Jolla, CA). Biofilm formation was compared using an unpaired t test. Complement mediated killing was evaluated as a log-fold reduction in *G. parasuis* between heat inactivated GPS and GPS and analyzed using an unpaired t test. Phagocytosis was evaluated using log-fold reduction in *G. parasuis* between the inoculum and PAM incubated wells. The difference in reduction between HS069 and HS069Δcap was compared using an ordinary one-way ANOVA. Comparison of adherence to porcine alveolar macrophages was completed using unpaired t tests comparing both bacterial cells per 3D4/31 cell and fluorescent intensity per 3D4/31 cell. Welch's corrections were used to account for differences in standard deviation when necessary. Results were considered significant at a p-value of $p < 0.05$.

Results

Development and confirmation of HS069 capsule mutant

The capsule locus of HS069 was removed by deleting the sequence from *neuA_3* to *etk* (alternatively *wzc* or *wzs*) (Figure 1-A), which removed the biosynthesis and glycosyltransferase proteins contained within the serovar 5 capsule locus. The deleted sequence was confirmed with whole genome sequencing using the PacBio sequencing platform. When the wild type and HS069Δcap genomes were compared, no HS069Δcap reads mapped to the region of the capsule locus (Figure 1-B).

Transmission electron microscopy (TEM) was also performed to phenotypically confirm the deletion of the capsule locus. In Figure 2, the surface of the HS069 wild type cells is irregular and thickened as compared to HS069Δcap. This confirmed the absence of capsular polysaccharide of the cell surface of HS069Δcap.

Growth characteristics and cellular morphology

Comparison of the growth kinetics of *G. parasuis* HS069 and HS069Δcap grown in BHI+ broth indicated there was no alteration in cellular proliferation associated with deletion of the capsule locus (Supplemental Figure 1).

Biofilm formation

Evaluation of static biofilm production indicated similar capacity for *G. parasuis* isolates to produce biofilm at all starting cell densities tested (0.05, 0.125, and 0.25) (data not shown). Statistical evaluation of static biofilm production by HS069 and HS069Δcap grown from an initial cell density of 0.05 indicated a significant enhancement in production associated with loss of capsular polysaccharide production ($p = 0.0193$) (Figure 3).

Complement mediated killing

Sensitivity to complement mediated killing (serum sensitivity) was evaluated using non-treated and heat inactivated GPS. A significant increase in sensitivity to complement killing was noted for HS069Δcap as compared to wild type HS069 ($p = 0.0207$) (Figure 4).

Adherence capacity to porcine alveolar macrophage cell line

Confocal microscopy was used to evaluate the adherence capacity of HS069 and HS069Δcap to porcine alveolar macrophages (3D4/31 cells). A distinct difference in the pattern of attachment was visualized between HS069 and HS069Δcap (Figure 5), with HS069Δcap producing aggregates of bacteria (Figure 6B) that were not noted with HS069 wild type (Figure 6A). Because of the aggregation of HS069Δcap, adherence was evaluated both as a bacterial count per 3D4/31 cell and fluorescent intensity per 3D4/31 cell. There was no statistically significant difference in adherence capacity when HS069 and HS069Δcap were compared for bacterial counts ($p = 0.0594$) or compared for fluorescent intensity ($p = 0.4296$).

Phagocytosis by primary porcine alveolar macrophages

Changes in susceptibility to phagocytosis were assessed by incubation with isolated primary porcine alveolar macrophages. After one hour of incubation, there was no difference in phagocytosis between HS069 and HS069Δcap ($P = 0.93$); however, after two hours of incubation, significantly more HS069Δcap were phagocytosed compared with HS069 wild type ($P < 0.01$) (Figure 7).

Virulence assessment

To assess the virulence of HS069Δcap as compared to the parent strain, a total of 11 CDCD pigs were challenged with HS069Δcap and four with wild type HS069. The parent strain resulted in 100% mortality by day 2 post-challenge, while animals challenged with HS069Δcap showed no clinical signs of *G. parasuis* infection and survived until the end of the study (20 days post-challenge).

Colonization and immune stimulation

A second study was conducted to evaluate nasal colonization of HS069Δcap in CDCD pigs. The presence of *G. parasuis* in nasal wash samples was assessed by PCR. Species specific primers detected *G. parasuis* on days 1 (1 pig) and 3 (2 pigs) post-challenge; however, all pigs were negative by PCR by day 5 post-challenge.

Serum antibody titer for animals challenged with HS069Δcap was determined at day 0, 7, 14, and 21 post-challenge (Figure 8). A mild increase in serum antibody to HS069Δcap sonicate was seen over the study period with the average Log_{10} titer rising from 3.121±0.336 to 3.572±0.250. However, upon intranasal challenge of HS069Δcap inoculated animals with wild type *G. parasuis* HS069, all animals succumbed to disease by 3 days post challenge.

Discussion

Capsular polysaccharide is an important factor for the survival and virulence of many bacteria. It is known to function in adhesion and immune evasion through resistance to complement killing and phagocytosis (11). In this study, we sought to better evaluate the function of capsule for *G. parasuis*, the causative agent of Glässer's Disease in pigs. Capsule has been correlated with virulence, with some serovars associated with disease and others with nasal colonization (3, 5, 6). The importance of capsule for *G. parasuis* was previously investigated in the serovar 5 strain SH0165 (10). Wang and colleagues found SH0165ΔcapD to be less serum resistant and non-virulent in a pig challenge, compared to the highly virulent parent strain (10). To confirm and expand on these findings and better understand how capsule contributes to *G. parasuis* disease, we generated a capsular mutant of the virulent serovar 5 strain HS069 followed by evaluation of sensitivity to complement killing and macrophage adhesion *in vitro* as well as virulence, colonization, and immune stimulation *in vivo*.

Here, the virulence of HS069Δcap was assessed with a combination of *in vitro* assays and an *in vivo* challenge. We found that, similar to previous reports, HS069Δcap was markedly less virulent in a pig challenge than the encapsulated parent strain. This confirmed what was noted by Wang and colleagues with SH0165Δcap (10). Reduced virulence of capsule deficient isolates has been attributed in part to the marked increase in serum sensitivity in non-encapsulated *G. parasuis* strains, which was seen with both HS069Δcap (Figure 4) and SH0165Δcap (10). Resistance to complement killing enables bacteria that penetrate mucosal defenses to better survive and disseminate to systemic sites, such as the central nervous system, joints, and serosal surfaces, as seen in *G. parasuis* infection.

Virulence has also been correlated with susceptibility to porcine alveolar macrophages

(28), which serve as the first line of defense during pulmonary infection. To investigate the interaction between HS069Δcap and porcine alveolar macrophages, we examined the adherence capacity of HS069Δcap and the parent strain to 3D4/31 cells (Figure 6). In this investigation, HS069 and HS069Δcap adhered equivalently, indicating the capsule is not playing a role in the interaction between *G. parasuis* HS069 and 3D4/31 cells. This analysis was made more complicated by the aggregation of HS069Δcap (Figure 5); however, through analysis of fluorescent intensity, we were able to account for aggregated bacteria (Figure 6B). We also evaluated HS069 and HS069Δcap for susceptibility to phagocytosis by primary PAMs, which revealed significantly more HS069Δcap was phagocytosed over two hours than HS069 wild type. This indicates the importance of *G. parasuis* capsule in protection against macrophage phagocytosis, which has not been shown previously in *G. parasuis*.

Additionally, we investigated the persistence of *G. parasuis* HS069Δcap in the nasal passageways, which indicated the importance of capsular polysaccharide not just in systemic infection, but also to colonization. Though biofilm production was enhanced for HS069Δcap (Figure 3), it did not contribute to persistent nasal colonization and HS069Δcap was cleared from all pigs in the second study by 5 days post-inoculation. This contrasts with colonization of the parent strain, which can persist in the nasal tract of vaccinated pigs (4/5 animals) through 11 days post-challenge (data not shown). Previous results assessing the role of capsule in colonization and adherence of other bacterial species have conflicted, with some studies reporting deficient colonization seen with capsular mutants and others indicating an increased capacity for adhesion to cell lines *in vitro* (12, 29-32). It has been hypothesized that the absence of capsule may increase the exposure of surface adhesins that contribute to adherence *in vitro*; however, most studies evaluated only *in vitro* adherence and have not investigated the effect of capsule in colonization *in vivo*. Our findings in this study indicate capsule may play a significant role in persistent colonization with *G. parasuis in vivo*, similar to that seen previously with *Streptococcus pneumoniae* (12).

The lack of virulence seen with *G. parasuis* capsular mutants would potentially make them a good candidate for an attenuated live vaccine; however, our investigation showed only a mild increase in *G. parasuis* serum antibody titer over the 21 day study period (Figure 8) with an average Log_{10} titer at 21 days post-inoculation of 3.572±0.250. The titers seen in animals inoculated with HS069Δcap are low when compared with other studies in which animals were vaccinated with a single dose HS069 bacterin (average Log_{10} titer 21 days post vaccination 4.221±0.377) or after boost vaccination (average Log_{10} day 42 titer 4.752±0.190), which has shown protection against homologous challenge. Low titers associated with HS069Δcap are likely due to its rapid clearance from the nasal cavity, which would limit interaction with immune cells and development of an adaptive immune response. Furthermore, when pigs inoculated with HS069Δcap were challenged with the parent strain, we saw no protection from *G. parasuis* disease, indicating the antibody response generated was not protective.

It is important to note, this evaluation of the importance of capsule in *G. parasuis* colonization and infection involved the generation and use of a mutant deficient in all genes of the capsule locus. Because of this, we cannot eliminate the possibility that there are alternative functions for genes within the capsule locus that may be contributing to the phenotypes we observed in this study. Additionally, because of the size of the capsule locus, a mutant complemented with the deleted genes was not generated and comparisons can only be made between the wild type and the mutant.

Conclusions

This investigation of the *G. parasuis* capsule mutant HS069Δcap confirms the importance of capsule to a fully virulent phenotype *in vivo* that has been seen previously with SH0615ΔcapD. Capsular polysaccharide plays an important role in resistance to complement killing and phagocytosis, which may be a key factor in the dissemination of *G. parasuis* during infection to systemic sites. In this study, we also found capsule to be an essential factor in *G. parasuis* HS069 for persistent colonization of the swine nasal cavity. However, because of the rapid clearance of HS069Δcap from the nasal cavity, generation of antibody was minimal and no protection was provided against challenge with the parent strain making HS069Δcap a poor modified live vaccine candidate.

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Primer	Sequence (5'-3')[a]	SH0165 genome (15)
P1 (neuAupFor1)	AAGACTGAGCTCTCGTTTTCCAGACAGCAATG	49398-49417
P2 (neuAupRev1)	AAGACTGGATCCCTCCTTTACATGCCCCCATC	50044-50025
P3 (wzsDnFor1)	AAGACTGGATCCTTGATGTAAGCGGTGGGATT	64033-64052
P4 (wzsDnRev1)	AAGCGAGTCGACAGTTGCGGCATAATCCAAAT	64763-64744
P5 (catFor)	GCGATGGATCCTGTGGAATTGTGAGCGGATA	n/a
P6 (catRev)	GCGATGGATCCACAAGCGGTTTCAACTAACGG	n/a

Table 1: Primers utilized in the construction of HS069Δcap.

[a] Restriction sites are underlined, BamHI: GGATCC, SalI: GTCGAC and SacI: GAGCTC.

Figure 1. Capsule locus deletion and verification. The entirety of the capsule locus was deleted from *neuA_3* to *etk* as indicated (A). The loss of the capsule locus was verified using Artemis Compare Tool (B). No sequence reads mapped to the region of the capsule locus.

Figure 2. Transmission electron microscopy visualization of capsule. The capsule layer of *G. parasuis* HS069 wild type (A) and HS069Δcap (B) were visualized using transmission electron microscopy to verify HS069Δcap was deficient in capsular polysaccharide production. The surface of HS069Δcap lacked thickened, irregular surface associated with capsule production.

Figure 3. Biofilm production by HS069 wild type and HS069Δcap. The capacity of *G. parasuis* HS069 and HS069Δcap to produce biofilm under static growth conditions was quantified using microtiter assays. Results here represent data from replicates with a starting OD_{600} of 0.05. The average absorbance at 538nm is shown (column) with standard deviation

indicated (error bars). Statistical significance is indicated by the asterisk (*) at a level of $p < 0.05$.

Figure 4. Evaluation of sensitivity to complement killing. *G. parasuis* HS069 and HS069Δcap were screened for resistance to complement mediated killing. Statistical analysis of the log10 reduction in CFU/mL was analyzed and statistical significance is indicated by the asterisk (*) at a level of $p < 0.05$.

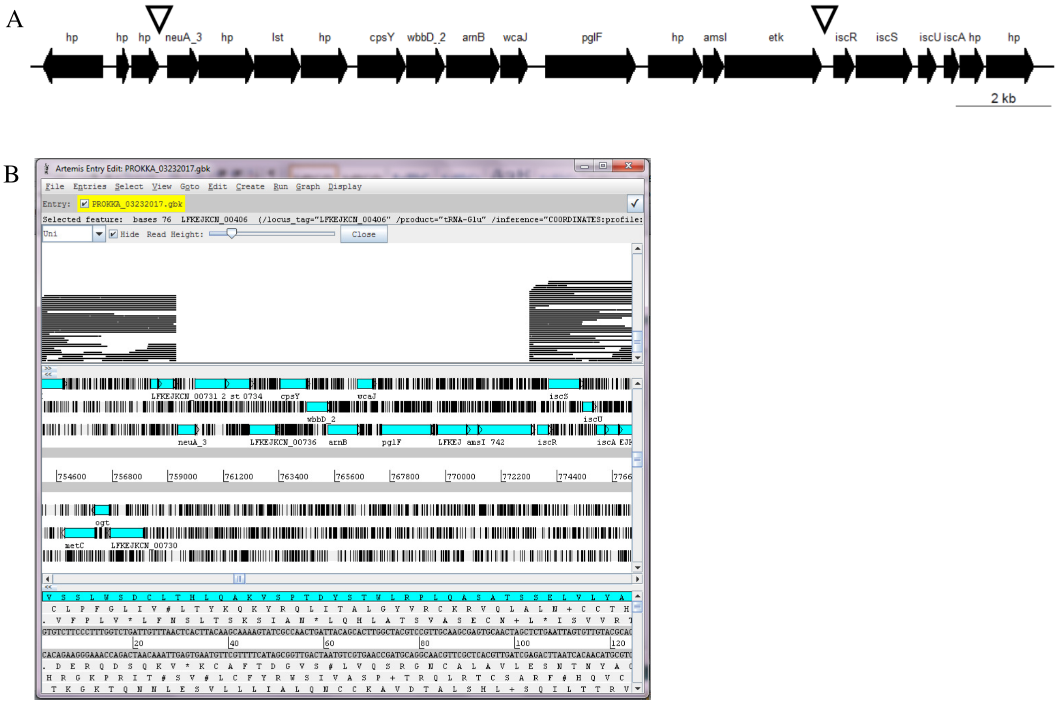
Figure 5. Adherence capacity of HS069 and HS069Δcap to 3D4/31 cells. The capacity for HS069 and HS069Δcap to adhere to porcine alveolar macrophages was evaluated using the 3D4/31 cell line. The interaction between *G. parasuis* and 3D4/31 cells was evaluated by confocal microscopy in chamber slides. *G. parasuis* strains were stained using a monocolonal antibody to the outer membrane protein P5. Bacterial aggregates were noted when evaluating HS069Δcap (B), but were not produced by the wild type HS069 isolate.

Figure 6. Adherence capacity to porcine alveolar macrophages (3D4/31 cell line). The capacity of *G. parasuis* HS069 and HS069Δcap to adhere to porcine alveolar macrophages was evaluated using the 3D4/31 cell line. Due to the clusters of bacterial cells noted using confocal microscopy, adherence was evaluated both as bacterial cells detected per 3D4/31 cell (A) and fluorescent intensity per 3D4/31 cell (B). No statistical differences were noted in bacteria per cell or fluorescent intensity per cell.

Figure 7. Evaluation of susceptibility to phagocytosis. *G. parasuis* HS069 and HS069Δcap were screened for susceptibility to phagocytosis using porcine alveolar macrophages. Log fold

reduction in *G. parasuis* [$\log_{10}$(CFU/mL)] is represented for both time points (1 hour and 2 hour incubation). The reduction of *G. parasuis* HS069 and HS069Δcap was not statistically different after 1 hour of incubation ($P = 0.93$); however, after 2 hours of incubation with PAMs significantly more *G. parasuis* HS069Δcap was phagocytosed than HS069 wild type ($P < 0.01$).

Figure 8. Serum antibody titers for pigs inoculated with HS069Δcap. The serum antibody titer was detected using an ELISA to whole cell sonicate. The data presented in this graph represent the animals in the second study investigating colonization with HS069Δcap.



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.  D  E  R  Q  D  S  Q  K  V  *  K  C  A  F  T  D  G  V  S  #  L  V  Q  S  R  G  N  C  A  L  A  V  L  E  S  N  T  N  Y  A
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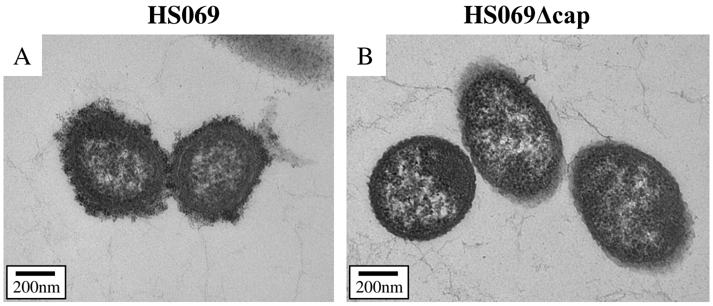
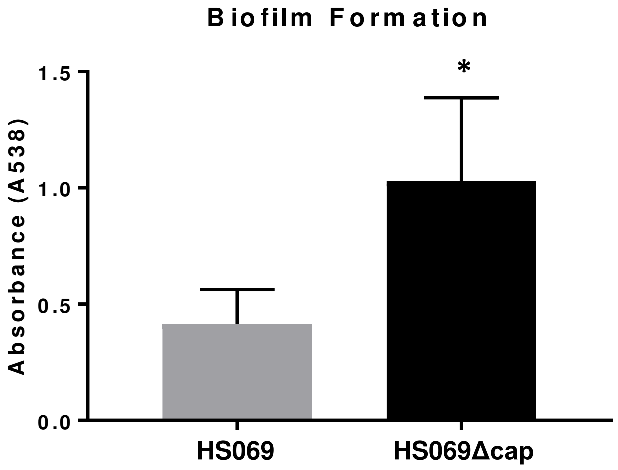
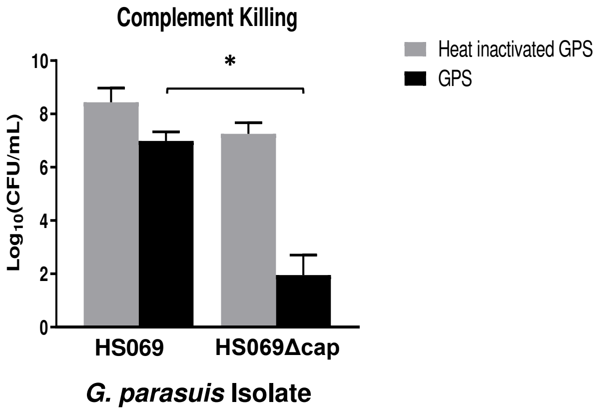
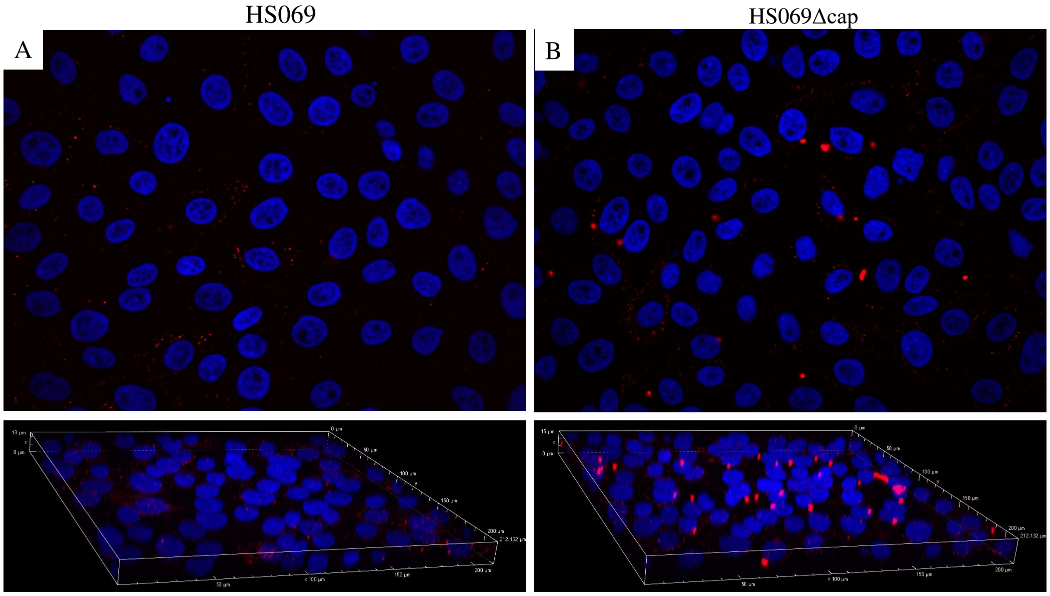
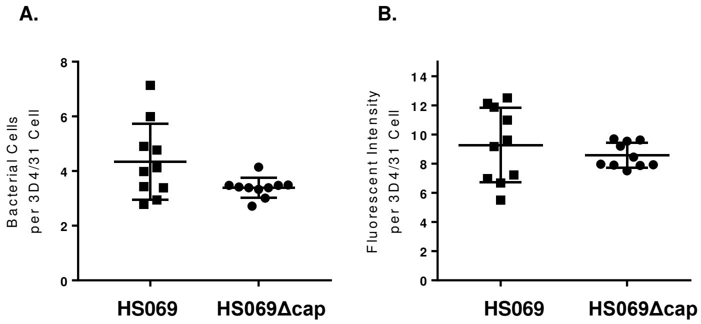
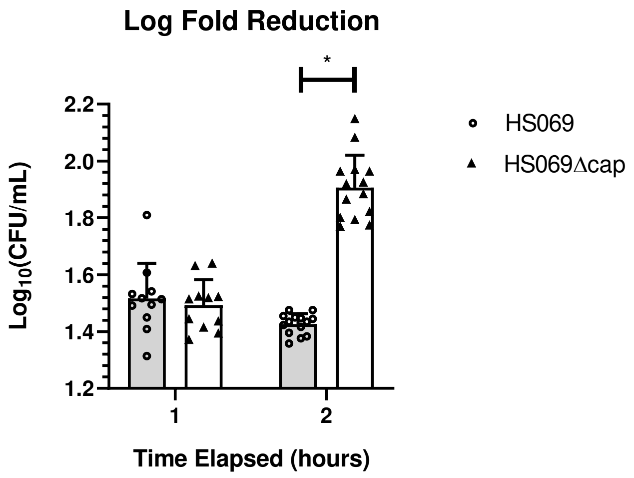

HS069
HS069Δcap
A
200nm
B
200nm

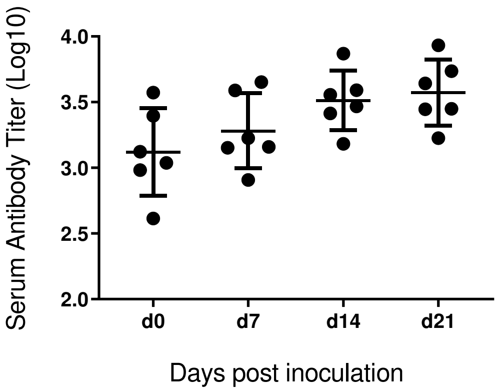

Biofilm Formation
Absorbance (A538)
1.5
1.0
0.5
0.0
*
HS069
HS069Δcap


Complement Killing
Heat inactivated GPS
GPS
*
10
8
6
4
2
0
Log₁₀(CFU/mL)
HS069
HS069Δcap
G. parasuis Isolate


HS069
HS069Δcap
A
B


A.
Bacterial Cells per 3D4/31 Cell
0
2
4
6
8
HS069
HS069Δcap
B.
Fluorescent Intensity per 3D4/31 Cell
0
2
4
6
8
10
12
14
HS069
HS069Δcap


Log Fold Reduction
*
2.2
2.0
1.8
1.6
1.4
1.2
Log$_{10}$(CFU/mL)
1
2
Time Elapsed (hours)
HS069
HS069Δcap


Serum Antibody Titer (Log10)
4.0
3.5
3.0
2.5
2.0
d0
d7
d14
d21
Days post inoculation