

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

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

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24 **Abstract**

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

37

38 Introduction

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

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

54 The functions of capsule have been partially evaluated in *G. parasuis* SH0165, a virulent
55 serovar 5 strain (10). The capsular mutant (SH0165 Δ capD) was significantly less virulent than
56 the wild type bacteria and unable to cause invasive disease in challenged pigs (10). SH0165 Δ cap
57 was also highly susceptible to complement mediated killing as compared to the wild type
58 bacteria (10). This presented evidence for the importance of capsule in causing invasive disease;
59 however, the characteristics of the capsular mutant were not fully elucidated and the capacity of
60 SH0165 Δ cap to colonize the swine nasal cavity was not evaluated. In this study, a capsule

61 mutant of *G. parasuis* HS069 (HS069 Δ cap), a virulent serovar 5 strain, was used to examine the
62 *in vitro* and *in vivo* characteristics of capsule. We evaluated HS069 Δ cap for susceptibility to
63 complement killing, biofilm formation, attachment to porcine macrophage 3D4/31 cells, and
64 phagocytosis by porcine alveolar macrophages. In addition, swine were challenged with HS069
65 and HS069 Δ cap to evaluate virulence, capacity for nasal colonization, and stimulation of host
66 immunity.
67

68 **Materials and Methods**

69 **Bacterial isolates**

70 The virulent serovar 5 *G. parasuis* strain HS069 was isolated from the lung of a pig with clinical
71 signs of respiratory disease (13). Production and verification of HS069 Δ cap is described within
72 the following mutant generation and results sections. *G. parasuis* strains were grown on brain
73 heart infusion (BHI) plates or BHI broth (Becton, Dickinson and Company, Franklin Lakes, NJ)
74 supplemented with 0.1 mg/mL nicotinamide adenine dinucleotide (NAD⁺) and 10% horse serum
75 (referred to as BHI+). *G. parasuis* strains were also grown on chocolate agar, made with
76 Columbia Blood Agar Base (Thermo Fisher Scientific Inc., Waltham, MA) supplemented 7%
77 defibrinated horse blood (TCS Bioscience Ltd., Botolph Claydon, England) lysed at 80°C for 10
78 minutes and 25 μ g/mL NAD⁺. Media were supplemented with chloramphenicol (1 μ g/mL) for
79 selection purposes. All strains were grown at 37°C with 5% CO₂.

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

83 **Mutant construction**

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

90 Biolabs. Plasmid DNA was extracted from the agarose gel using Gel Extraction Kit (Qiagen).
91 DNA concentrations were measured using a NanoDrop (Bio-Rad Laboratories, Hercules, CA).
92 *Construction of plasmid pGEMT-neuAup-Cm-wzsdn*: Plasmid pGEMT-neuAup-cat-wzsdn was
93 constructed for deletion of the whole capsule gene cluster using a three-step cloning strategy.
94 First, genomic DNA of *G. parasuis* isolate HS069 was used as a template to amplify the
95 upstream and downstream regions flanking the 14-kb capsule locus (14, 15). The 647-bp
96 upstream region of the *neuA* gene (*neuAup*) was amplified using primers P1 and P2 and the 731-
97 bp downstream region of the *wzs* gene (*wzsdn*) using primers P3 and P4 (Table 1). In parallel,
98 the chloramphenicol resistance cassette (*Cm*), containing the 9-bp DNA uptake signal sequence
99 (USS) of 5'-ACCGCTTGT (16) was amplified from 50 ng linearized plasmid pUSScat (17) as
100 the template with primers P5 and P6 (Table 1). Second, PCR products were digested and ligated
101 into the pGEM-T vector. The PCR product of the upstream region of the *neuA* gene was digested
102 with SacI /BamHI and the downstream region of the *wzs* gene was digested with Sall/ BamHI.
103 The pGEM-T vector was digested with SacI /Sall. Restricted products were gel purified and
104 ligated. The constructed plasmid was transformed into *E. coli* TOP10 and transformants were
105 confirmed using PCR. After purification, the plasmid pGEMT-neuAup-wzsdn was verified by
106 Sanger sequencing. Finally, the purified plasmid pGEMT-neuAup-wzsdn and PCR product of
107 the *Cm* cassette were subject to BamHI restriction. The gel purified fragments were mixed,
108 ligated and transformed into *E. coli* TOP10. The resulting plasmid pGEMT-neuAup-cat-wzsdn
109 was extracted and confirmed by Sanger sequencing.
110 *Construction of the whole capsule locus deletion mutant Δ cap::*Cm* mutant of *G. parasuis**
111 *HS069*: The plasmid pGEMT-neuAup-cat-wzsdn was linearized with SacI and used to transform
112 *G. parasuis* HS069 using natural transformation method as described previously with some

113 modifications (18). Briefly, HS069 was grown on chocolate agar overnight and suspended in
114 BHI broth to achieve an $OD_{660} = 2$. Then, a 20 μ l aliquot of a 1/10 dilution of the suspension was
115 spread in a 10 mm area on prewarmed chocolate agar and 20 μ l of 8 mM cAMP and 10 μ l of
116 donor DNA in TE buffer were added and mixed with the bacterial cells. The mixture was
117 incubated at 37°C overnight. Bacterial cells were scraped up, suspended in 300 μ l BHI broth, and
118 plated onto chocolate agar with 1 μ g/mL chloramphenicol. After incubation at 37°C for 2 days,
119 suspected transformants were verified using PCR. For negative control, 10 μ l of TE buffer
120 without donor DNA was added to a bacterial spot. The deletion was also confirmed by whole
121 genome sequencing using the Illumina HiSeq 250 platform and PacBio RS II Resequencing
122 protocol of the SMRT Analysis software v.2.3.0. Assembly of the PacBio generated HS069 wild
123 type was done using HGAP (19), circularized using Circlator v.1.1.3 (20), and polished using
124 Quiver v.1. The Illumina reads were subsequently mapped onto the PacBio assembly to correct
125 small indels. Comparison of HS069 Δ cap against the wild type was made by mapping the
126 Illumina and PacBio reads of HS069 Δ cap against the finished HS069 wild type assembly.

127 **Transmission electron microscopy (TEM) for capsule visualization**

128 Capsule was visualized via transmission electron microscopy (TEM) using previously described
129 methods (21-23). Briefly, *G. parasuis* grown on BHI+ plates overnight were suspended in 0.1 M
130 cacodylate buffer with 2.5% glutaraldehyde and 0.1% ruthenium red and incubated for 2 hours at
131 room temperature. Bacteria were pelleted and resuspended in 0.1 M cacodylate buffer with 2.5%
132 glutaraldehyde and 1.0 mg/mL of polycationic ferritin and incubated for 30 minutes at room
133 temperature. Bacteria were washed three times in 0.1 M cacodylate buffer. After staining with
134 ruthenium red and ferritin, samples were post-fixed with 2% osmium tetroxide and rinsed three
135 times in 0.1 M cacodylate buffer. The samples were processed through graded ethanols,

136 propylene oxide, and embedded in Eponate 12 (Ted Pella Inc., Redding, CA). Following a 48
137 hour polymerization, thin sections were taken and stained with 4% uranyl acetate and Reynolds'
138 lead stain. Sections were examined with a FEI Tecnai G² BioTWIN electron microscope (FEI
139 Co., Hillsboro, OR).

140 **Growth kinetics**

141 Growth kinetics of *G. parasuis* HS069 and HS069 Δ cap were evaluated using a GeneQuant Pro
142 spectrophotometer (Amersham PLC, Little Chalfont, United Kingdom). *G. parasuis* isolates
143 were inoculated from a liquid overnight culture into BHI+ broth at an OD₆₀₀ of 0.05. The
144 cultures were incubated at 37°C with 5% CO₂ and shaking at 200 RPM. OD₆₀₀ readings were
145 taken hourly for 8 hours.

146 **Biofilm assay using microtiter plate**

147 The microtiter plate assay for biofilm formation was adapted from a protocol described by Cassat
148 et al. (24). Overnight cultures of *G. parasuis* were adjusted to an initial OD₆₀₀ of 0.05, 0.125, or
149 0.25 in BHI+ broth. Diluted *G. parasuis* was plated in triplicate on a Nunc 96-well flat bottom
150 plate (Thermo Fisher Scientific Inc., Waltham, MA) and incubated statically for 48 hours at 37°C
151 with 5% CO₂. Cultures were gently removed from the plate and washed three times with sterile
152 PBS. Biofilm was fixed with 100% ethanol, allowed to dry for 10 minutes, and stained with
153 0.1% crystal violet. After 15 minutes at room temperature, plates were washed gently three times
154 with PBS and allowed to dry overnight. Crystal violet was eluted from the biofilm with 150 μ L
155 of 100% ethanol for 10 minutes. The elution (120 μ L) was transferred to a new 96 well plate and
156 absorbance measured at 538 nm with a SpectraMax M5 (Molecular Devices, LLC, Sunnyvale,
157 CA). Three independent replicates were performed.

158 **Complement mediated killing**

159 To assess sensitivity to complement mediated killing, *G. parasuis* cultures were treated with
160 guinea pig serum (GPS) (Quidel, San Diego, CA). Heat inactivated GPS (30 minutes at 56°C)
161 was used as a negative control. *G. parasuis* cultures were grown on BHI+ plates and suspended
162 in PBS to reach an OD₆₀₀ of 0.42 (1x10⁸ bacteria/mL). In a 96-well plate, 90 uL of GPS was
163 added to 10 uL of *G. parasuis* (approximately 10⁶ CFU). The *G. parasuis* and GPS incubated for
164 1 hour at 37°C, 5% CO₂, 100 rpm shaking. Serial dilutions were plated on BHI+ plates.

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

166 The interaction of *G. parasuis* HS069 and HS069Δcap with porcine alveolar macrophages was
167 tested *in vitro* using the 3D4/31 cell line (ATCC, Manassas, VA). 3D4/31 cells were maintained
168 in complete Advanced RPMI 1640 (Thermo Fisher Scientific Inc., Waltham, MA) as per
169 ATCC's recommendations. For the assay, 3D4/31 cells were plated into 4-well chamber slides
170 (ibidi USA Inc., Madison, WI) at 5x10⁵ cells/mL and allowed to adhere overnight. *G. parasuis*
171 HS069 and HS069Δcap were added to the chambers to obtain an MOI of 10:1 and incubated for
172 1 hour at 37°C and 5% CO₂. After incubating, 3D4/31 cells were washed to remove non-adherent
173 bacteria and chamber slides were placed on ice. *G. parasuis* cells were incubated with mouse
174 monoclonal antibody to the outer membrane protein P5 (provided by M. Gottschalk) for 30
175 minutes at 4°C followed by incubation with goat anti-mouse IgG3 (SouthernBiotech,
176 Birmingham, AL) for 30 minutes at 4°C. Cells were fixed with ice cold 50:50 methanol:acetone
177 for 10 minutes and dried. Images were taken using Nikon AR1+Si confocal microscope and
178 evaluated using the NIS Elements software (Nikon Instruments Inc., Melville, NY). Bacterial
179 intensity was evaluated using 10 random views.

180 **Phagocytosis assessment using primary porcine alveolar macrophages**

181 Porcine alveolar macrophages (PAMs) were isolated as previously described (25, 26), with

182 modifications. Briefly, the lungs of healthy pigs were flushed with PBS repeatedly until around
183 250 mL of fluid was collected. The lavage fluid was centrifuged at 1000 RPM for 10 minutes.
184 The pellet was washed twice and resuspended in complete RPMI-1640 medium (10% fetal
185 bovine serum, 1 $\mu\text{g}/\text{mL}$ fungizone, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin). PAMs
186 were allowed to adhere to petri dishes for 2 hours at 37° C with 5% CO₂. After 2 hours, media
187 and non-adherent cells were aspirated and adherent cells were washed with complete RPMI.
188 Adherent cells were removed via cell scraping, washed twice with PBS, and resuspended in
189 RPMI without antibiotics. Cells were counted and scored for viability using the Countess II
190 Automated Cell Counter (Invitrogen, Carlsbad, CA). PAMs were plated into 48-well plates with
191 5×10^5 PAMs per well and allowed to adhere for 20 minutes prior to starting the assay.

192 Bacterial stocks were generated by suspending agar grown HS069 and HS069 Δcap in
193 PBS with 50% glycerol at an OD₆₀₀ of 0.42. The stocks were quantified via serial dilution and
194 frozen at -80° C until use. HS069 or HS069 Δcap stocks were diluted to obtain an MOI of 10:1 in
195 250 μL total volume per well (approximately 5×10^6 CFU/well).

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

202 ***G. parasuis* challenge**

203 All experiments were approved by the National Animal Disease Center's Institutional Animal
204 Care and Use Committee. Caesarian-derived, colostrum-deprived (CDCD) pigs were derived at

205 the National Animal Disease Center. At 4 weeks of age, pigs were intranasally challenged with 2
206 mL (1 mL per nostril) of 1×10^8 CFU/mL *G. parasuis* HS069 (4 pigs) or HS069 Δ cap (5 pigs)
207 suspended in PBS. Pigs were monitored for clinical signs (lameness, respiratory distress,
208 lethargy, neurologic signs) and humanely euthanized when systemic signs of disease were noted.
209 At necropsy, gross lesions were recorded and serum, nasal swabs, serosal swabs, joint fluid, lung
210 lavage, and cerebral spinal fluid samples were obtained and plated for CFU counts. Serum and
211 nasal swab samples were taken on day 0 and day 21 post-challenge. To investigate the vaccine
212 potential of HS069 Δ cap, the pigs surviving challenge with HS069 Δ cap (5 pigs) were intranasally
213 challenged with 2 mL (1 mL per nostril) of 1×10^8 of *G. parasuis* HS069 wild type on day 21
214 post-challenge with HS069 Δ cap. Pigs were monitored and treated as described above

215 A follow up study was conducted to evaluate nasal colonization of HS069 Δ cap in CDCD
216 pigs. At 6 weeks of age, 6 pigs were inoculated intranasally with 2 mL (1 mL per nostril) of
217 1×10^8 CFU/mL suspension of *G. parasuis* HS069 Δ cap in PBS. Nasal swabs were obtained on
218 day 0, 1, 3, 5, 7, and 14 post-challenge for *G. parasuis* detection. *G. parasuis* species specific
219 PCR was run on these samples to detect bacterial colonization utilizing the primer set described
220 by Howell et al. (27). Briefly, DNA was extracted from 50 μ L of nasal swab samples using the
221 MagMAX Pathogen RNA/DNA Kit (Thermo Fisher Scientific Inc., Waltham, MA). Extracted
222 DNA was screened as previously described using *G. parasuis* species specific primers:
223 HPS_219690793-F (5'-ACAACCTGCAAGTACTTATCGGGAT-3') and HPS_219690793-R
224 (5'-TAGCCTCCTGTCTGATATTCCCACG-3') (27).

225 **ELISA for serum antibody titer**

226 Nunc MaxiSorp plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with 0.5
227 mg/mL of HS069 Δ cap sonicate in 100 mM carbonate-bicarbonate buffer (pH 9.6) at 4°C

228 overnight. Plates were washed with 0.05% Tween 20 in PBS (PBST) followed by blocking for 1
229 hour at 37°C with 2% bovine serum albumin (BSA) in PBST. After washing with PBST, serum
230 samples were serially diluted in 1% BSA/PBST and applied to wells for 2 hours at 37°C. Plates
231 were washed with PBST and HRP-conjugated secondary goat anti-swine IgG antibody (SeraCare
232 Life Sciences, Milford, MA) was diluted 1:50,000 in 1% BSA/PBST added and incubated for 1
233 hour at 37°C. ELISAs were developed using tetramethylbenzidine (TMB) substrate (Thermo
234 Fisher Scientific Inc., Waltham, MA). TMB was added to each well, incubated at room
235 temperature for 15 minutes, and the reaction halted with sulfuric acid (2 N). Absorbance 450 nm
236 was measured on a SpectraMax M5 (Molecular Devices, LLC, Sunnyvale, CA).

237 **Statistical analysis**

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

248

249 **Results**

250 **Development and confirmation of HS069 capsule mutant**

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

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

261 **Growth characteristics and cellular morphology**

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

265 **Biofilm formation**

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

271 **Complement mediated killing**

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

275 **Adherence capacity to porcine alveolar macrophage cell line**

276 Confocal microscopy was used to evaluate the adherence capacity of HS069 and HS069 Δ cap to
277 porcine alveolar macrophages (3D4/31 cells). A distinct difference in the pattern of attachment
278 was visualized between HS069 and HS069 Δ cap (Figure 5), with HS069 Δ cap producing
279 aggregates of bacteria (Figure 6B) that were not noted with HS069 wild type (Figure 6A).

280 Because of the aggregation of HS069 Δ cap, adherence was evaluated both as a bacterial count per
281 3D4/31 cell and fluorescent intensity per 3D4/31 cell. There was no statistically significant
282 difference in adherence capacity when HS069 and HS069 Δ cap were compared for bacterial
283 counts ($p = 0.0594$) or compared for fluorescent intensity ($p = 0.4296$).

284 **Phagocytosis by primary porcine alveolar macrophages**

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

290 **Virulence assessment**

291 To assess the virulence of HS069 Δ cap as compared to the parent strain, a total of 11 CDCD pigs
292 were challenged with HS069 Δ cap and four with wild type HS069. The parent strain resulted in
293 100% mortality by day 2 post-challenge, while animals challenged with HS069 Δ cap showed no
294 clinical signs of *G. parasuis* infection and survived until the end of the study (20 days post-

295 challenge).

296 **Colonization and immune stimulation**

297 A second study was conducted to evaluate nasal colonization of HS069 Δ cap in CDCD pigs. The
298 presence of *G. parasuis* in nasal wash samples was assessed by PCR. Species specific primers
299 detected *G. parasuis* on days 1 (1 pig) and 3 (2 pigs) post-challenge; however, all pigs were
300 negative by PCR by day 5 post-challenge.

301 Serum antibody titer for animals challenged with HS069 Δ cap was determined at day 0, 7,
302 14, and 21 post-challenge (Figure 8). A mild increase in serum antibody to HS069 Δ cap sonicate
303 was seen over the study period with the average Log₁₀ titer rising from 3.121 \pm 0.336 to
304 3.572 \pm 0.250. However, upon intranasal challenge of HS069 Δ cap inoculated animals with wild
305 type *G. parasuis* HS069, all animals succumbed to disease by 3 days post challenge.

306

307 **Discussion**

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

320 Here, the virulence of HS069 Δ cap was assessed with a combination of *in vitro* assays and
321 an *in vivo* challenge. We found that, similar to previous reports, HS069 Δ cap was markedly less
322 virulent in a pig challenge than the encapsulated parent strain. This confirmed what was noted by
323 Wang and colleagues with SH0165 Δ cap (10). Reduced virulence of capsule deficient isolates has
324 been attributed in part to the marked increase in serum sensitivity in non-encapsulated *G.*
325 *parasuis* strains, which was seen with both HS069 Δ cap (Figure 4) and SH0165 Δ cap (10).
326 Resistance to complement killing enables bacteria that penetrate mucosal defenses to better
327 survive and disseminate to systemic sites, such as the central nervous system, joints, and serosal
328 surfaces, as seen in *G. parasuis* infection.

329 Virulence has also been correlated with susceptibility to porcine alveolar macrophages

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

341 Additionally, we investigated the persistence of *G. parasuis* HS069 Δ cap in the nasal
342 passageways, which indicated the importance of capsular polysaccharide not just in systemic
343 infection, but also to colonization. Though biofilm production was enhanced for HS069 Δ cap
344 (Figure 3), it did not contribute to persistent nasal colonization and HS069 Δ cap was cleared from
345 all pigs in the second study by 5 days post-inoculation. This contrasts with colonization of the
346 parent strain, which can persist in the nasal tract of vaccinated pigs (4/5 animals) through 11
347 days post-challenge (data not shown). Previous results assessing the role of capsule in
348 colonization and adherence of other bacterial species have conflicted, with some studies
349 reporting deficient colonization seen with capsular mutants and others indicating an increased
350 capacity for adhesion to cell lines *in vitro* (12, 29-32). It has been hypothesized that the absence
351 of capsule may increase the exposure of surface adhesins that contribute to adherence *in vitro*;
352 however, most studies evaluated only *in vitro* adherence and have not investigated the effect of

353 capsule in colonization *in vivo*. Our findings in this study indicate capsule may play a significant
354 role in persistent colonization with *G. parasuis in vivo*, similar to that seen previously with
355 *Streptococcus pneumoniae* (12).

356 The lack of virulence seen with *G. parasuis* capsular mutants would potentially make
357 them a good candidate for an attenuated live vaccine; however, our investigation showed only a
358 mild increase in *G. parasuis* serum antibody titer over the 21 day study period (Figure 8) with an
359 average Log₁₀ titer at 21 days post-inoculation of 3.572±0.250. The titers seen in animals
360 inoculated with HS069Δcap are low when compared with other studies in which animals were
361 vaccinated with a single dose HS069 bacterin (average Log₁₀ titer 21 days post vaccination
362 4.221±0.377) or after boost vaccination (average Log₁₀ day 42 titer 4.752±0.190), which has
363 shown protection against homologous challenge. Low titers associated with HS069Δcap are
364 likely due to its rapid clearance from the nasal cavity, which would limit interaction with
365 immune cells and development of an adaptive immune response. Furthermore, when pigs
366 inoculated with HS069Δcap were challenged with the parent strain, we saw no protection from
367 *G. parasuis* disease, indicating the antibody response generated was not protective.

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

375 **Conclusions**

376 This investigation of the *G. parasuis* capsule mutant HS069 Δ cap confirms the importance
377 of capsule to a fully virulent phenotype *in vivo* that has been seen previously with
378 SH0615 Δ capD. Capsular polysaccharide plays an important role in resistance to complement
379 killing and phagocytosis, which may be a key factor in the dissemination of *G. parasuis* during
380 infection to systemic sites. In this study, we also found capsule to be an essential factor in *G.*
381 *parasuis* HS069 for persistent colonization of the swine nasal cavity. However, because of the
382 rapid clearance of HS069 Δ cap from the nasal cavity, generation of antibody was minimal and no
383 protection was provided against challenge with the parent strain making HS069 Δ cap a poor
384 modified live vaccine candidate.
385

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401

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511
- 512

Primer	Sequence (5'-3') ^a	SH0165 genome (15)
P1 (neuAupFor1)	AAGACT <u>GAGCTCT</u> CGTTTTCCAGACAGCAATG	49398-49417
P2 (neuAupRev1)	AAGACT <u>GGATCC</u> CTCTTACATGCCCCATC	50044-50025
P3 (wzsDnFor1)	AAGACT <u>GGATCCT</u> TGATGTAAGCGGTGGGATT	64033-64052
P4 (wzsDnRev1)	AAGCGAGTCGACAGTTGCGGCATAATCCAAAT	64763-64744
P5 (catFor)	GCGAT <u>GGATCCT</u> GTGGAATTGTGAGCGGATA	n/a
P6 (catRev)	GCGAT <u>GGATCC</u> ACAAGCGGTTCAACTAACGG	n/a

513 Table 1: Primers utilized in the construction of HS069 Δ cap.

514 ^a Restriction sites are underlined, BamHI: GGATCC, Sall: GTCGAC and SacI: GAGCTC.

515

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

519

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

524

525 **Figure 3. Biofilm production by HS069 wild type and HS069 Δ cap.** The capacity of *G.*
 526 *parasuis* HS069 and HS069 Δ cap to produce biofilm under static growth conditions was
 527 quantified using microtiter assays. Results here represent data from replicates with a starting
 528 OD₆₀₀ of 0.05. The average absorbance at 538nm is shown (column) with standard deviation

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

530

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

535

536 **Figure 5. Adherence capacity of HS069 and HS069 Δ cap to 3D4/31 cells.** The capacity for
537 HS069 and HS069 Δ cap to adhere to porcine alveolar macrophages was evaluated using the
538 3D4/31 cell line. The interaction between *G. parasuis* and 3D4/31 cells was evaluated by
539 confocal microscopy in chamber slides. *G. parasuis* strains were stained using a monoclonal
540 antibody to the outer membrane protein P5. Bacterial aggregates were noted when evaluating
541 HS069 Δ cap (B), but were not produced by the wild type HS069 isolate.

542

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

549

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

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

556

557 **Figure 8. Serum antibody titers for pigs inoculated with HS069 Δ cap.** The serum antibody
558 titer was detected using an ELISA to whole cell sonicate. The data presented in this graph
559 represent the animals in the second study investigating colonization with HS069 Δ cap.















