

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 $\Delta$ cap) through allelic exchange following natural transformation. HS069 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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 $\Delta$ capD) was significantly less virulent than  
56 the wild type bacteria and unable to cause invasive disease in challenged pigs (10). SH0165 $\Delta$ 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 $\Delta$ cap to colonize the swine nasal cavity was not evaluated. In this study, a capsule

61 mutant of *G. parasuis* HS069 (HS069 $\Delta$ cap), a virulent serovar 5 strain, was used to examine the  
62 *in vitro* and *in vivo* characteristics of capsule. We evaluated HS069 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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<sup>+</sup>) 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  $\mu$ g/mL NAD<sup>+</sup>. Media were supplemented with chloramphenicol (1  $\mu$ g/mL) for  
79 selection purposes. All strains were grown at 37°C with 5% CO<sub>2</sub>.

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  $\mu$ g/mL) or chloramphenicol (50  $\mu$ 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  $\Delta$ 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  $\mu$ l aliquot of a 1/10 dilution of the suspension was  
115 spread in a 10 mm area on prewarmed chocolate agar and 20  $\mu$ l of 8 mM cAMP and 10  $\mu$ 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  $\mu$ l BHI broth, and  
118 plated onto chocolate agar with 1  $\mu$ g/mL chloramphenicol. After incubation at 37°C for 2 days,  
119 suspected transformants were verified using PCR. For negative control, 10  $\mu$ 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 $\Delta$ cap against the wild type was made by mapping the  
126 Illumina and PacBio reads of HS069 $\Delta$ 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 G<sup>2</sup> BioTWIN electron microscope (FEI  
139 Co., Hillsboro, OR).

#### 140 **Growth kinetics**

141 Growth kinetics of *G. parasuis* HS069 and HS069 $\Delta$ 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 OD<sub>600</sub> of 0.05. The  
144 cultures were incubated at 37°C with 5% CO<sub>2</sub> and shaking at 200 RPM. OD<sub>600</sub> 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 OD<sub>600</sub> 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% CO<sub>2</sub>. 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  $\mu$ L  
155 of 100% ethanol for 10 minutes. The elution (120  $\mu$ 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 OD<sub>600</sub> of 0.42 (1x10<sup>8</sup> bacteria/mL). In a 96-well plate, 90 uL of GPS was  
163 added to 10 uL of *G. parasuis* (approximately 10<sup>6</sup> CFU). The *G. parasuis* and GPS incubated for  
164 1 hour at 37°C, 5% CO<sub>2</sub>, 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 5x10<sup>5</sup> 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% CO<sub>2</sub>. 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% CO<sub>2</sub>. 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  $5 \times 10^5$  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 $\Delta\text{cap}$  in  
193 PBS with 50% glycerol at an OD<sub>600</sub> of 0.42. The stocks were quantified via serial dilution and  
194 frozen at -80° C until use. HS069 or HS069 $\Delta\text{cap}$  stocks were diluted to obtain an MOI of 10:1 in  
195 250  $\mu\text{L}$  total volume per well (approximately  $5 \times 10^6$  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 $\Delta\text{cap}$  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  $1 \times 10^8$  CFU/mL *G. parasuis* HS069 (4 pigs) or HS069 $\Delta$ 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 $\Delta$ cap, the pigs surviving challenge with HS069 $\Delta$ cap (5 pigs) were intranasally  
213 challenged with 2 mL (1 mL per nostril) of  $1 \times 10^8$  of *G. parasuis* HS069 wild type on day 21  
214 post-challenge with HS069 $\Delta$ cap. Pigs were monitored and treated as described above

215 A follow up study was conducted to evaluate nasal colonization of HS069 $\Delta$ cap in CDCD  
216 pigs. At 6 weeks of age, 6 pigs were inoculated intranasally with 2 mL (1 mL per nostril) of  
217  $1 \times 10^8$  CFU/mL suspension of *G. parasuis* HS069 $\Delta$ 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  $\mu$ 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 $\Delta$ 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 $\Delta$ 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 $\Delta$ cap genomes were compared, no HS069 $\Delta$ 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 $\Delta$ cap. This confirmed the absence of capsular  
260 polysaccharide of the cell surface of HS069 $\Delta$ cap.

### 261 **Growth characteristics and cellular morphology**

262 Comparison of the growth kinetics of *G. parasuis* HS069 and HS069 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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 $\Delta$ cap to  
277 porcine alveolar macrophages (3D4/31 cells). A distinct difference in the pattern of attachment  
278 was visualized between HS069 and HS069 $\Delta$ cap (Figure 5), with HS069 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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 $\Delta$ cap ( $P = 0.93$ ); however, after two hours of  
288 incubation, significantly more HS069 $\Delta$ cap were phagocytosed compared with HS069 wild type  
289 ( $P < 0.01$ ) (Figure 7).

#### 290 **Virulence assessment**

291 To assess the virulence of HS069 $\Delta$ cap as compared to the parent strain, a total of 11 CDCD pigs  
292 were challenged with HS069 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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 $\Delta$ cap was determined at day 0, 7,  
302 14, and 21 post-challenge (Figure 8). A mild increase in serum antibody to HS069 $\Delta$ cap sonicate  
303 was seen over the study period with the average Log<sub>10</sub> titer rising from 3.121 $\pm$ 0.336 to  
304 3.572 $\pm$ 0.250. However, upon intranasal challenge of HS069 $\Delta$ 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 SH0165ΔcapD 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 HS069Δcap was assessed with a combination of *in vitro* assays and  
321 an *in vivo* challenge. We found that, similar to previous reports, HS069Δcap 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 SH0165Δcap (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 HS069Δcap (Figure 4) and SH0165Δcap (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 $\Delta$ cap and porcine alveolar macrophages, we examined the adherence  
332 capacity of HS069 $\Delta$ cap and the parent strain to 3D4/31 cells (Figure 6). In this investigation,  
333 HS069 and HS069 $\Delta$ 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 $\Delta$ 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 $\Delta$ cap for susceptibility to phagocytosis by primary PAMs, which  
338 revealed significantly more HS069 $\Delta$ 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 $\Delta$ 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 $\Delta$ cap  
344 (Figure 3), it did not contribute to persistent nasal colonization and HS069 $\Delta$ 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 Log<sub>10</sub> 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 Log<sub>10</sub> titer 21 days post vaccination  
362 4.221±0.377) or after boost vaccination (average Log<sub>10</sub> 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 $\Delta$ cap confirms the importance  
377 of capsule to a fully virulent phenotype *in vivo* that has been seen previously with  
378 SH0615 $\Delta$ 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 $\Delta$ cap from the nasal cavity, generation of antibody was minimal and no  
383 protection was provided against challenge with the parent strain making HS069 $\Delta$ cap a poor  
384 modified live vaccine candidate.  
385

386 **Acknowledgements**

387 The authors would like to thank Sarah Shore for sharing her expertise on microtiter biofilm  
388 assays. This research was funded by the U. S. Department of Agriculture. Mention of trade  
389 names or commercial products in this article is solely for the purpose of providing specific  
390 information and does not imply recommendation or endorsement by the USDA. USDA is an  
391 equal opportunity provider and employer. This work was supported by a Longer and Larger  
392 (LoLa) grant from the Biotechnology and Biological Sciences Research Council (BBSRC grant  
393 numbers BB/G020744/1, BB/G019177/1, BB/G019274/1 and BB/G018553/1), the UK  
394 Department for Environment, Food and Rural Affairs, and Zoetis awarded to the Bacterial  
395 Respiratory Diseases of Pigs-1 Technology (BRaDP1T) consortium. LAW is funded by a Sir  
396 Henry Dale Fellowship co-funded by the Royal Society and Wellcome Trust (Grant Number:  
397 109385/Z/15/Z). This project was additionally supported in part by an appointment to the  
398 Agricultural Research Service Postdoctoral Research Program administered by the Oak Ridge  
399 Institute for Science and Education (ORISE). Funding sources had no role in study design, data  
400 collection and analysis, decision to publish, or preparation of the manuscript.

401

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512

Primer	Sequence (5'-3') <sup>a</sup>	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>GGATCC</u> TTGATGTAAGCGGTGGGATT	64033-64052
P4 (wzsDnRev1)	AAGCGAGTCGACAGTTGCGGCATAATCCAAAT	64763-64744
P5 (catFor)	GCGAT <u>GGATC</u> CTGTGGAATTGTGAGCGGATA	n/a
P6 (catRev)	GCGAT <u>GGATCC</u> ACAAGCGGTTTCAACTAACGG	n/a

513 Table 1: Primers utilized in the construction of HS069Δcap.

514 <sup>a</sup> 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 HS069Δcap (B) were visualized using transmission electron  
 522 microscopy to verify HS069Δcap was deficient in capsular polysaccharide production. The  
 523 surface of HS069Δcap lacked thickened, irregular surface associated with capsule production.

524

525 **Figure 3. Biofilm production by HS069 wild type and HS069Δcap.** The capacity of *G.*  
 526 *parasuis* HS069 and HS069Δcap to produce biofilm under static growth conditions was  
 527 quantified using microtiter assays. Results here represent data from replicates with a starting  
 528 OD<sub>600</sub> 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 $\Delta$ cap  
532 were screened for resistance to complement mediated killing. Statistical analysis of the log<sub>10</sub>  
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 $\Delta$ cap to 3D4/31 cells.** The capacity for  
537 HS069 and HS069 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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 $\Delta$ cap was phagocytosed than HS069 wild type ( $P < 0.01$ ).

556

557 **Figure 8. Serum antibody titers for pigs inoculated with HS069 $\Delta$ 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 $\Delta$ cap.















