Veterinary Microbiology 305 (2025) 110509

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Capsular immunity is necessary for protection against some but not all strains of *Glaesserella parasuis*

Samantha J. Hau [a], Shi-Lu Luan [b], Lucy A. Weinert [b], Paul R. Langford [c], Andrew Rycroft [d], Brendan W. Wren [e], Duncan J. Maskell [b], Alexander W. (Dan) Tucker [b], Susan L. Brockmeier [a,*]

[a] National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, IA, United States
[b] Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK
[c] Section of Paediatrics, Department of Medicine, Imperial College London, St. Mary's Campus, London, UK
[d] The Royal Veterinary College, Hawkshead Campus, Hatfield, United Kingdom
[e] Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK

ARTICLE INFO

Keywords:
Glaeserella parasuis
Glasser's disease
Vaccine
Bacterin
Swine

ABSTRACT

Glaesserella parasuis is the causative agent of Glässer's disease in pigs and results in significant losses to the swine industry annually. Due to the serovar and strain specific response associated with many bacterin vaccines, there has been difficulty generating broad heterologous protection. Here, an unencapsulated *G. parasuis* mutant (HS069Δcap) was assessed as a bacterin vaccine and compared to a bacterin made from the encapsulated parent strain, against challenge with the homologous, parent strain (serovar 5) as well as four heterologous challenge strains (serovar 1, 4, 5, and 14). Both the HS069 and HS069Δcap bacterins generated high titers to the homologous and heterologous strains. The HS069Δcap bacterin was able to provide protection against the parent strain as well as 12939 (serovar 1), 2170B (serovar 4), and MN-H (serovar 13), but was unable to protect animals from challenge with Nagasaki (serovar 5). In contrast, the HS069 bacterin was able to provide protection against all challenge strains, showing the importance of serovar specific immunity against the challenge strain Nagasaki. This appears to be due to the production of a more abundant and well-organized capsule in Nagasaki as compared to HS069. This study indicates HS069Δcap is a good candidate strain for bacterin development; however, it may be less able to provide protection against highly encapsulated strains of *G. parasuis*.

1. Introduction

Glaesserella parasuis is a Gram-negative bacterium from the *Pasteurellaceae* family and the causative agent of Glässer's disease in pigs. Glässer's disease is a systemic inflammatory condition characterized by fibrinopurulent polyserositis, polyarthritis, and meningitis, which contributes to post-weaning mortality in pigs (Aragon et al., 2019). *G. parasuis* is also commonly found in the nasal cavity of healthy pigs and is considered a component of the normal nasal microbiota (Aragon et al., 2019).

Isolates of *G. parasuis* are characterized by their capsular polysaccharide into 15 serovars (Kielstein and Rapp-Gabrielson, 1992). This is done through serologic typing methods or using PCR to identify the genes within the capsule locus to designate the specific capsule type (Howell et al., 2017). Capsule is an important virulence factor in *G. parasuis*, with capsule deficient mutants being avirulent in swine infection models (Eberle et al., 2020; Wang et al., 2013). Although the correlation is not exact, there also seems to be a relationship between serovar and virulence and some serovars are more commonly associated with systemic disease, while others are most commonly found within the nasal cavity of healthy animals (Aragon et al., 2010; Macedo et al., 2021; Oliveira et al., 2003).

Control of Glässer's disease has been challenging due to the lack of heterologous protection between strains. While many vaccine platforms have been evaluated, including bacterin vaccines, avirulent live vaccines, modified live vaccines, subunit vaccines, and vesicle based vaccines (Brockmeier et al., 2013; Martin de la Fuente et al., 2009; McCaig et al., 2016; Miniats et al., 1991a; Miniats et al., 1991b; Nielsen, 1993; Smart and Miniats, 1989), it has been challenging to induce broadly protective immunity. Bacterin based vaccine platforms have shown good protection against challenge with the homologous strain; however, heterologous protection is not consistently found using bacterin

* Correspondence to: 1920 Dayton Ave, Ames, IA 50010, United States.
E-mail address: Susan.Brockmeier@usda.gov (S.L. Brockmeier).

https://doi.org/10.1016/j.vetmic.2025.110509
Received 21 March 2023; Received in revised form 17 March 2025; Accepted 7 April 2025
Available online 11 April 2025

vaccination (Bak and Riising, 2002; Miniats et al., 1991a; Miniats et al., 1991b; Smart and Miniats, 1989). This has been attributed to the development of immunity to the serovar of the bacterin strain, which often is unable to provide protection against heterologous strains.

Here, we utilized a previously developed capsule deficient mutant (HS069Δcap) to evaluate the role of capsular immunity in protection against *G. parasuis* (Eberle et al., 2020). This was assessed by employing both HS069Δcap and the parent strain (HS069) as whole cell bacterins and utilizing homologous and heterologous challenge with five diverse *G. parasuis* isolates.

2. Materials and methods

2.1. Bacterial isolates and growth conditions

G. parasuis isolates utilized in this study can be found in Table 1. Isolates were grown in brain heart infusion broth (BD Biosciences, Franklin Lakes, NJ) supplemented with 10 % heat inactivated horse serum and 0.1 mg/mL NAD (BHI+) or BHI+ agar. All cultures were incubated at 37° C with 5 % CO_2.

2.2. Bacterin generation

G. parasuis HS069 and HS069Δcap were streaked onto BHI+ agar from freezer stocks. Isolates were re-streaked onto 10–15 BHI+ plates. Lawn cultures were harvested into PBS from the plates after overnight growth. Serial dilutions were made of the cell suspension to quantify colony forming units (CFU)/mL prior to inactivation. The cultures were inactivated overnight at room temperature with the addition of 10 % buffered formalin to a final concentration of 0.25 % formalin. Sterility was checked by plating 100 μL onto BHI+ agar.

After inactivation was verified, a quantity of the bacterial suspension to obtain 10^9 CFU/dose was washed with PBS, pelleted, and resuspended in PBS to comprise 80 % of the total vaccine volume. Emulsigen D (MVP Laboratories, Omaha, NE) was added at 20 % of the vaccine volume as an adjuvant. Each vaccine dose consisted of a 2 mL volume containing 10^9 CFU and 20 % Emulsigen D.

2.3. Animal vaccination and challenge

All animal work was approved by the Institutional Animal Care and Use Committee at the National Animal Disease Center. Vaccine efficacy was tested in two independent trials. Animal trial 1 consisted of 29 cesarean-derived, colostrum-deprived (CDCD) pigs obtained from Struve Labs Inc. (Manning, IA). Animal trial 2 consisted of 45 CDCD pigs obtained from Struve Labs Inc. Pigs were vaccinated intramuscularly on day 0 and day 21 of the study with HS069 bacterin, HS069Δcap bacterin, or phosphate buffered saline (PBS) with 20 % Emulsigen D (adjuvant only). On day 42, animals were challenged intranasally with 2 mL of 1×10^8 of *G. parasuis* wild type HS069 (animal trial 1), 12939 (animal trial 1), Nagasaki (animal trial 2), MN-H (animal trial 2), or 2170B (animal trial 2). Each vaccine/challenge group consisted of five pigs except the adjuvant only/HS069 challenged group, which had only four pigs. Pigs were monitored for signs of systemic disease (lameness, lethargy, respiratory disease, neurologic signs) and humanely euthanized when severe clinical signs were noted. Surviving pigs were euthanized 11–12 days after challenge. At necropsy, gross findings were noted and the following samples were collected and screened for *G. parasuis* via plating on BHI+ agar: nasal swab, serum, serosal swab, joint sample, cerebrospinal fluid (CSF) sample, and lung lavage.

Table 1
G. parasuis isolates used in vaccination and challenge.

Isolate Name	Isolate Serovar	Isolate Source	Reference
HS069	5	Lung of pig with Glässer's disease	(Howell et al., 2014)
HS069Δcap	Capsule deficient	Previously generated mutant	(Eberle et al., 2020)
12939	1	Lung of pig with Glässer's disease	(Brockmeier et al., 2013; Brockmeier et al., 2014)
2170B	4	Joint of a pig with polyserositis	(Zehr et al., 2012; Zehr and Tabatabai, 2011)
MN-H	13	Pig with polyserositis	(Brockmeier et al., 2013; Brockmeier et al., 2014)
Nagasaki	5	Meninges of pig with septicemia/ meningitis	(Brockmeier et al., 2014; Kielstein and Rapp-Gabrielson, 1992)

2.4. IgG ELISA

Blood was collected into a BD Vacutainer serum separator tube (SST) (BD Biosciences, San Jose, CA). Serum was isolated as per manufacturer's recommendations and samples were stored at −80° C until use. Immulon-2 plates (Thermo Fisher Scientific, Waltham, MA) were coated with 100 μL of a 0.5 μg/mL solution of *G. parasuis* sonicate in 100 mM carbonate-bicarbonate buffer (pH 9.6) overnight at 4° C. Plates were blocked with 200 μL of blocking buffer (2 % bovine serum albumin [BSA] in PBS-0.05 % Tween 20 [PBST]) for two hours at room temperature then washed three times with PBST. Two-fold dilutions of test sera were generated in 1 % BSA in PBST and 100 μL of each dilution was added to duplicate wells and incubated at room temperature for two hours. Plates were washed three times with PBST and incubated for one hour in 100 μL of a 1:50,000 dilution of horseradish peroxidase conjugated anti-porcine IgG (SeraCare Life Sciences, Milford, MA). After washing with PBST, 100 μL of tetramethylbenzidine substrate (TMB) (Life Technologies, Carlsbad, CA) was added and the reaction was stopped after 10 min with 50 μL of 2 N H_2SO_4. The optical density at 450 nm with correction at 655 nm was read and the OD data was modeled as a nonlinear function of the $\log_{10}$ dilution using the log (agonist)-versus-response variable slope four-parameter logistic model in GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA). Endpoint titers were interpolated using 2 times the average OD of the negative control (gnotobiotic) serum sample as the cutoff.

2.5. Cytokine production by PBMCs

PBMCs were isolated on day 21 and 42 as previously described (Braucher et al., 2012). PBMCs were seeded into 96-well plates at 2.5×10^5 cells per well and stimulated with whole cell sonicate of HS069 or HS069Δcap at 0.16 μg/mL in a total volume of 250 μL. After 72 hours, culture supernatants were harvested and frozen at −80°C until performing assays.

Cytokines were quantified using antigen capture ELISAs. Plates were coated overnight with 50 μL anti-cytokine antibody diluted in 100 mM carbonate-bicarbonate buffer as indicated in Table S1. Prior to use, plates were washed with PBST and blocked with 100 μL 2 % BSA in PBST for 2 hours. Plates were washed and 50 μL of cell supernatant was added to duplicate wells for antigen capture. After incubation for 2 hours, plates were washed and probed for 1 hour with 50 μL biotinylated anti-cytokine antibody diluted in 1 % BSA in PBST as indicated in Table S1. Plates were washed and probed with 50 μL streptavidin-HRP conjugate diluted 1:20,000 in 1 % BSA in PBST for 1 hour. Plates were washed, developed with TMB, and stopped as described above. Plates were read as indicated above. Cytokine concentration was interpolated from a standard curve generated from dilutions of recombinant cytokine (2.0 ng/mL, 1.0 ng/mL, 0.5 ng/mL, 0.25 ng/mL, 0.125 ng/mL, and 0 ng/mL) using the average of the duplicate sample wells.

2.6. Hydrophobicity analysis

Capsule quantification was assessed using hydrophobicity measurements previously described by Rosenberg et al. with minor

modifications (Rosenberg et al., 1980). Briefly, bacteria were grown overnight on BHI+ agar and suspended in PUM buffer. In 10 mm round bottom test tubes, 1.2 mL bacterial suspension and 0.1 mL xylenes (Mallinckrodt Pharmaceuticals, Staines-upon-Thames, United Kingdom) were combined. The mixture was incubated at 37ºC for 10 minutes, vortexed for 2 minutes, and allowed to rest for 15 minutes at room temperature. The aqueous phase was harvested and OD_{400} was measured for the suspension pre- and post-treatment. Results were calculated as a change in OD_{400} after xylene treatment.

2.7. Transmission electron microscopy (TEM)

Capsule was assessed as previously described (Borrathybay et al., 2003; Jacques and Foiry, 1987; Kawamoto et al., 2007), with the modifications indicated by Eberle et. al (Eberle et al., 2020). *G. parasuis* was grown on BHI+ plates overnight and suspended in 0.1 M cacodylate buffer containing 2.5 % glutaraldehyde and 0.1 % ruthenium red. After a 2 h incubation, bacteria were pelleted and resuspended in 0.1 M cacodylate buffer containing 2.5 % glutaraldehyde and 1.0 mg/mL of polycationic ferritin for 30 min. Bacteria were washed in 0.1 M cacodylate buffer and post-fixed with 2 % osmium tetroxide. After rinsing in 0.1 M cacodylate buffer, samples were dried and embedded in Eponate 12 (Ted Pella Inc., Redding, CA). Samples were polymerized for 48 hours, sectioned, and stained with 4 % uranyl acetate and Reynolds' lead stain. Samples were examined using a FEI Tecnai G^2 BioTWIN electron microscope (FEI Co., Hillsboro, OR).

2.8. Statistics

Statistics were completed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA). Survival was analyzed using the Kaplan and Meier method and compared using the log-rank test. Log_{10} antibody titers and cytokine production were compared using two-way ANOVAs. Hydrophobicity data was analyzed using a one-way ANOVA. A P value of ≤ 0.05 was considered significant.

3. Results

3.1. ELISA titers

Collected serum was evaluated on days 0, 21, and 42 for antibody titers to HS069, HS069Δcap, and the challenge strain (12939, 2170B, MN-H, or Nagasaki). Titers to HS069 and HS069Δcap in bacterin vaccinated pigs were higher than that of adjuvant only vaccinated pigs in both trials on day 21 and 42 ($p < 0.01$, Figs. 1 and 2). Heterologous titers were also evaluated for the challenge strains. Significantly higher titers were found in the bacterin vaccinated groups as compared to the adjuvant only animals on day 21 and 42 for all heterologous challenge strains ($p < 0.01$, Figs. 1 and 2).

Comparison of titers from HS069 bacterin vaccinated animals and HS069Δcap bacterin vaccinated animals revealed similar titers to the challenge strains. In animal trial 1, animals vaccinated with the HS069 bacterin had similar titers to those vaccinated with the HS069Δcap bacterin for HS069, HS069Δcap, and 12939 on day 21 (Fig. 1-A-C); however, on day 42, vaccination with the HS069Δcap bacterin resulted in higher titers to HS069Δcap than HS069 bacterin vaccination (Fig. 1-B). Similarity in titers between bacterin vaccines was also seen in animal trial 2 when evaluating titers for HS069, HS069Δcap, 2170B, MN-H, and Nagasaki (Fig. 2-A-E). Statistically higher titers to HS069Δcap were seen in HS069Δcap bacterin vaccinated animals on day 21 as compared to HS069 bacterin vaccinated animals ($p = 0.026$, Fig. 2-B), though titers were comparable on day 42. Although they did not reach the statistical threshold, on day 42 the titer of HS069Δcap bacterin vaccinated animals was higher for 2170B ($p = 0.099$) and lower for MN-H ($p = 0.0865$) when compared with HS069 bacterin vaccinated animals (Fig. 2-C and -D, respectively).

3.2. Cell mediated immune response

Peripheral blood mononuclear cells (PBMCs) were harvested and stimulated with whole cell sonicates of HS069 and HS069Δcap to

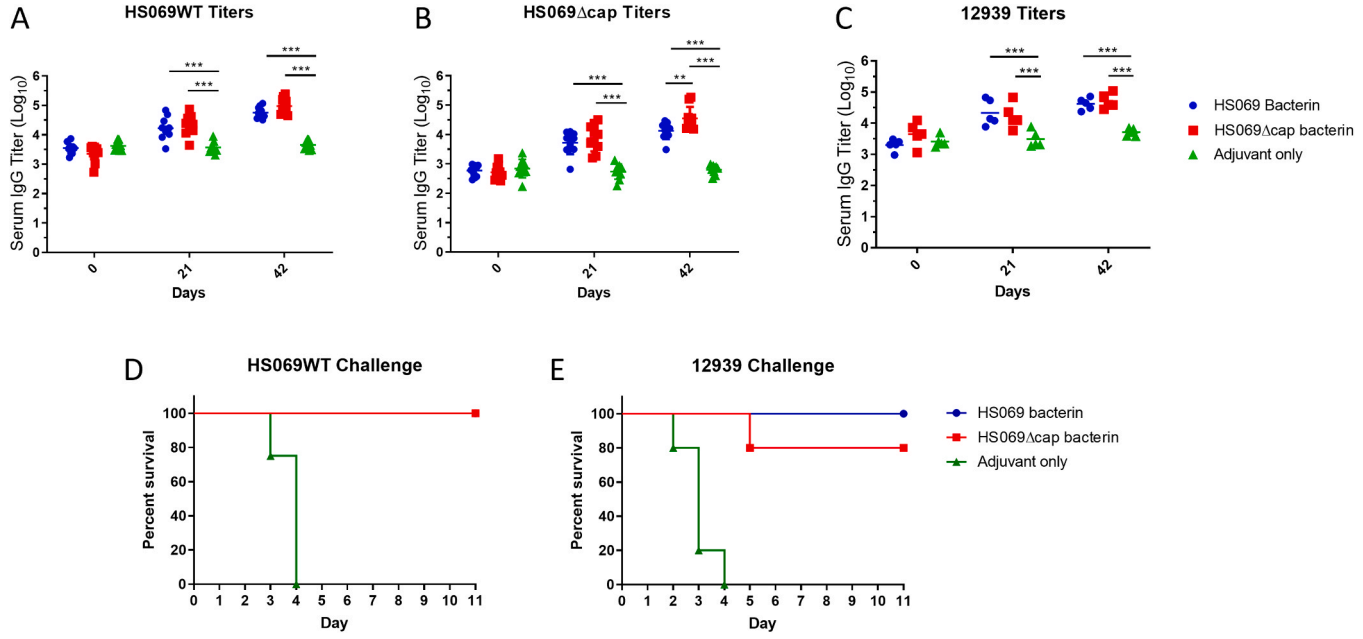

Fig. 1. Serum IgG response and survival from Experiment 1. Titers to HS069 (A), HS069Δcap (B), and 12939 (C) were elevated in both bacterin vaccinated groups as compared to the adjuvant only group on day 21 and 42. No difference in titer to HS069 or 12939 was found between HS069 and HS069Δcap bacterin vaccinated animals on day 21 or on day 42; however, higher titers to HS069Δcap were seen for HS069Δcap bacterin vaccinated animals at day 42 when compared with HS069 bacterin vaccinated animals. Survival was compared between groups following challenge with the homologous strain HS069 (D) and the heterologous strain 12939 (E). Improved survival was seen in bacterin vaccinated groups when compared with the sham vaccinated animals. No difference in survival was noted between the HS069 bacterin and the HS069Δcap bacterin. Statistical significance is indicated by the asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0001$).

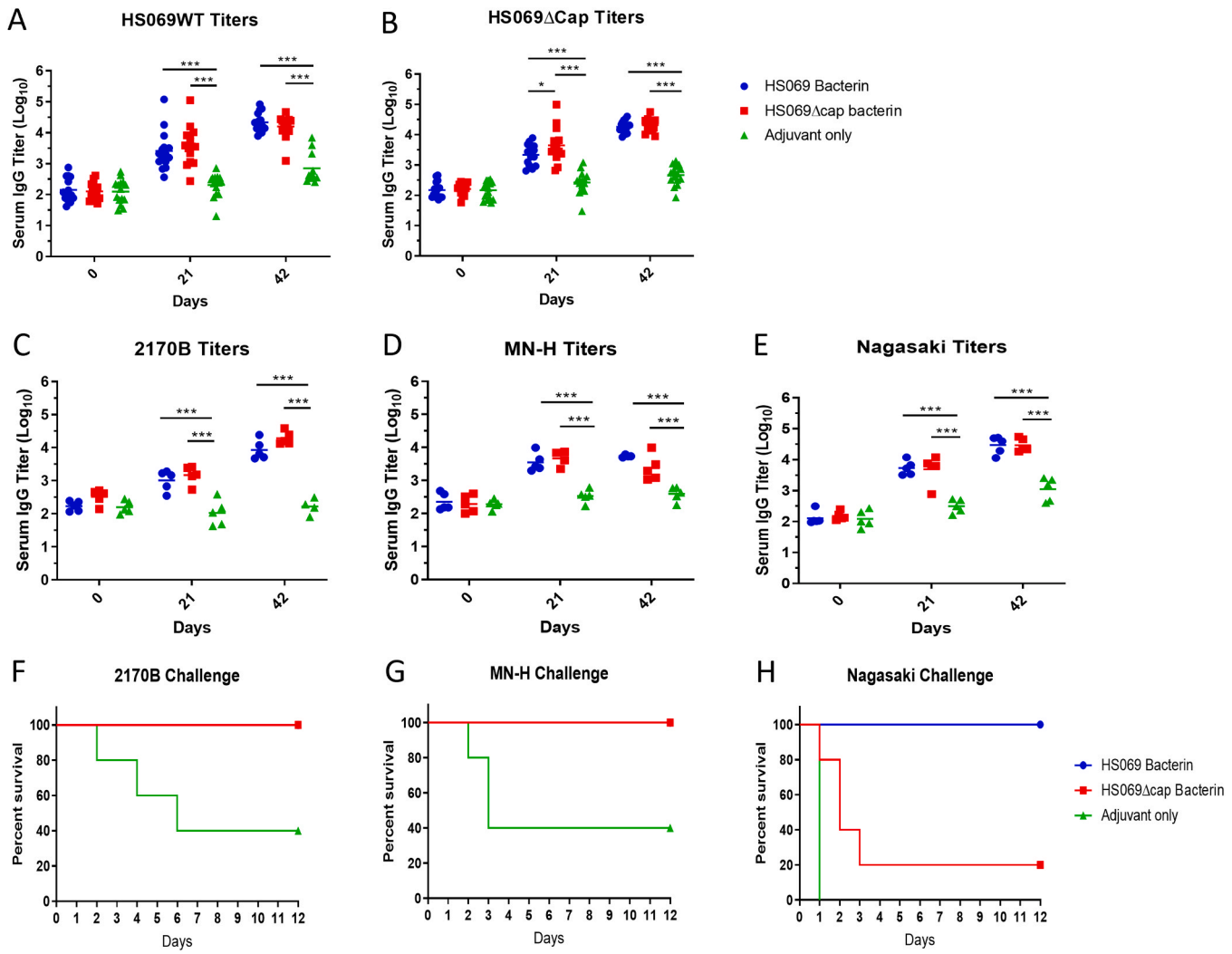

Fig. 2. Serum IgG response and survival from Experiment 2. Titers to HS069 (A), HS069Δcap (B), 2170B (C), MN-H (D), and Nagasaki (E) were elevated in both bacterin vaccinated groups as compared to the adjuvant only group on day 21 and 42 ($p < 0.001$). As noted in Experiment 1, titers were similar for HS069 and HS069Δcap bacterin vaccinated animals. Survival following challenge with the heterologous strains 2170B (F) and MN-H (G) was increased but not statistically significant ($p = 0.1667$); however, one pig from the adjuvant only group in both 2170B and MN-H challenges showed clinical signs consistent with *G. parasuis* disease. When assessing clinical disease rather than survival, protection becomes significant for both bacterin vaccines ($p = 0.0476$). Animals vaccinated with the HS069 bacterin were protected against challenge with the heterologous strain Nagasaki ($p = 0.0079$); however, pigs vaccinated with the HS069Δcap bacterin were not. Titers for panels A and B represent all vaccinated animals, while C-E represent only animals challenged with the corresponding strains. Statistical significance is indicated by the asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0001$).

compare the cell mediated immune response to *G. parasuis* after vaccination with the HS069 or HS069Δcap bacterin. Production of IFN-γ, IL2, IL10, IL13, IL17, and TNF-α were evaluated on day 21 and 42. No differences in cytokine production from PBMCs were identified on day 21 between pigs vaccinated with the HS069 bacterin, HS069Δcap bacterin, and adjuvant only (data not shown). On day 42, production of IL10 from the PBMCs of animals vaccinated with the HS069Δcap bacterin was higher than that of HS069 bacterin and adjuvant only vaccinated animals after stimulation with HS069Δcap ($p = 0.0376$ and 0.0218, respectively). Production of other assessed cytokines was comparable between HS069 and HS069Δcap bacterin vaccinated animals (data not shown).

3.3. Homologous challenge

The capacity of both the HS069 bacterin and the HS069Δcap bacterin to protect pigs against challenge with the homologous strain (HS069 wild type) was assessed. Bacterin vaccinated animals survived challenge with HS069, while adjuvant only animals succumbed to disease (Fig. 1-D) ($p = 0.0079$). Protection from disease was similar between animals receiving the HS069 bacterin or the HS069Δcap bacterin ($p > 0.99$).

3.4. Heterologous challenge

Animals were challenged with four heterologous strains with respect to serovar ($n = 3$) or strain ($n = 1$) following vaccination with HS069 (serovar 5) or HS069Δcap bacterins: 12939 (serovar 1), 2170B (serovar 4), MN-H (serovar 13), or Nagasaki (serovar 5). Survival curves of animals following heterologous challenge can be found in Fig. 1-E (12939) and Fig. 2-F-H (2170B, MN-H, and Nagasaki, respectively). Heterologous challenge with the serovar 1 strain 12939 (animal trial 1) showed significant differences between protection in bacterin vaccinated animals as compared to adjuvant only animals (Fig. 1-E) ($p = 0.0079$ and $p = 0.0476$, for the HS069 and HS069Δcap bacterins, respectively). There was no difference in protection between animals vaccinated with the HS069 bacterin and the HS069Δcap bacterin ($p > 0.99$). In animal trial 2, although all bacterin vaccinated pigs survived challenge with

2170B (serovar 4) (Fig. 2-F) and MN-H (serovar 13) (Fig. 2-G), the difference in survival between bacterin vaccinated and adjuvant only animals was not statistically different due to diminished lethality with these strains ($p = 0.1667$); however, in both groups, one adjuvant only pig showed clinical signs of *G. parasuis* disease that resolved without progression to severe disease. When considering animals with clinical signs of disease, there was a significant difference between the bacterin vaccinated and adjuvant only animals ($p = 0.0476$).

Challenge with Nagasaki, a homologous serovar but heterologous strain to the vaccine, produced different results from challenge with the other strains. Animals vaccinated with the wild type HS069 bacterin were protected from challenge with Nagasaki, while 80 % of animals (4/5) vaccinated with the HS069Δcap bacterin succumbed to *G. parasuis* disease ($p = 0.0476$) (Fig. 2-H). Statistical differences were noted in the survival curves of the adjuvant only animals and HS069Δcap bacterin vaccinated animals ($p = 0.0143$), though overall survival was not statistically different ($p > 0.99$).

3.5. Capsule quantification

The quantity of capsule produced by strains HS069 and Nagasaki was assessed using a hydrophobicity assay. The unencapsulated HS069Δcap was found to be the most hydrophobic strain, with the lowest percentage of the cell suspension remaining in the aqueous phase (Fig. 3-A). The HS069 parent strain was significantly less hydrophobic than the mutant strain ($p < 0.001$). Additionally, the heterologous serovar 5 isolate Nagasaki was significantly less hydrophobic than the wild type vaccine strain (HS069) ($p = 0.0317$). Because capsule is a hydrophilic substrate, increased encapsulation results in increased retention of cells within the aqueous phase (or a higher percent OD_{400} post-assay), as seen with HS069 and HS069Δcap (Fig. 3-A). Capsule structures of HS069 and Nagasaki were also imaged using electron microscopy to assess quantity and structure (Fig. 3-B-C). Strain Nagasaki demonstrated a more organized and thicker capsule layer (Fig. 3-B) compared to strain HS069 (Fig. 3-C). The capsular polysaccharide of HS069 appeared sparser and the external structure was more frayed than that seen in Nagasaki.

4. Discussion

G. parasuis is a significant contributor to post-weaning mortality in the swine industry. Because of the rapid progression of disease and the presence of multiple strains and serovars within the industry and farms (Aragon et al., 2019; Cerda-Cuellar et al., 2010), Glässer's disease has been challenging to control. Different vaccination strategies have been attempted; however, it is difficult to generate broadly protective immunity, which has been attributed to strain specific immunity and more specifically associated with capsular immunity that results in serovar specific protection (Aragon et al., 2019; Martin de la Fuente et al., 2009; Miniats et al., 1991a; Miniats et al., 1991b; Smart and Miniats, 1989). While the efficacy of capsular directed immunity in protection against encapsulated bacteria is well known (Croucher et al., 2018; Gasparini and Panatto, 2011; Kelly et al., 2004; Sadarangani, 2018), in this study we found immunity to capsule is not necessary for protection against all *G. parasuis* isolates.

Vaccination with both the parent strain HS069 and the defined mutant HS069Δcap bacterins generated high titers and provided protection against homologous and heterologous challenge strains. Due to the absence of capsular polysaccharide, antibody induced by the HS069Δcap bacterin would be directed at cellular proteins and cell wall components and would not generate capsular directed immunity. Survival of animals vaccinated with the HS069Δcap bacterin against challenge with HS069 indicates that immunity to capsular polysaccharide is not necessary for protection against all *G. parasuis* isolates. Additionally, the protection afforded by the HS069Δcap bacterin was able to provide heterologous protection against 12939 (serovar 1), 2170B (serovar 4), and MN-H (serovar 13); which indicates immunity to proteins and cell wall components can provide heterologous protection. In contrast, protection against Nagasaki, a heterologous serovar 5 isolate, was only seen in animals vaccinated with the HS069 bacterin. We speculate the difference in protection against Nagasaki between the HS069 and HS069Δcap bacterins could be due to the stimulation of capsule directed immunity. This would mean that, for some *G. parasuis* isolates, capsular immunity may be more important for survival. We observed differences in the median survival time between animals vaccinated with HS069Δcap bacterin and adjuvant only, which may indicate immunity to cellular proteins and the cell wall were able to slow the progression of disease. To better understand *in vivo* protection, future studies should characterize the bacterin induced immune response and assesses capsule specific immunity.

In this study, we speculated differences in survival between animals vaccinated with the HS069 and HS069Δcap bacterins after challenge with HS069 and Nagasaki, both serovar 5 isolates, were associated with differences in immunity to capsular polysaccharide. We hypothesized immunity to capsular polysaccharide was more important for protection against Nagasaki due to differences in capsule production between Nagasaki and HS069. More surface polysaccharide would prevent

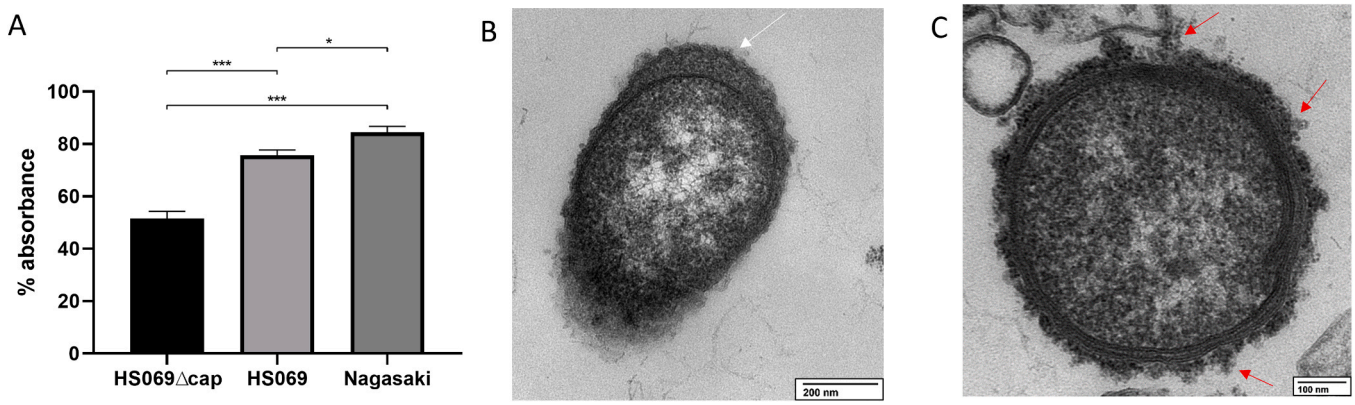

Fig. 3. Capsule assessment. (A) The affinity of each strain for xylenes was assessed to indicate the hydrophobicity of each individual strain. Results are reported as a percent of the absorbance at OD_{400} of the initial suspension. Because capsule is a hydrophilic substance, a greater portion of the suspension remains in the aqueous phase for highly encapsulated strains, resulting in a higher percent absorbance post-incubation. The outer membrane is hydrophobic and the unencapsulated HS069Δcap is removed by the hydrophobic xylenes phase, resulting in a lower percent absorbance post-incubation. Significant differences in hydrophobicity are represent by the asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$). TEM image of *G. parasuis* Nagasaki (B) and HS069 (C). Nagasaki produced capsular polysaccharide that generally appeared thicker and better organized (white arrow) while HS069 produced less capsular polysaccharide and the material appeared more frayed at the external surface (red arrows).

antibodies directed at protein and cell wall components from interacting with their cognate antigens, limiting the efficacy of those antibodies in protection. To assess our hypothesis, we evaluated capsular polysaccharide using a hydrophobicity assay and TEM. We found Nagasaki produced a thicker, more well-organized capsule, which may explain why protection was observed following vaccination with the HS069 bacterin as HS069 produces capsule and could stimulate capsule directed immunity.

Our findings in this study lend insight into previous work evaluating *G. parasuis* bacterin vaccines and may help clarify the differences seen in efficacy of bacterin vaccination against heterologous challenge. Previous work from our lab compared the use of Nagasaki and HS069 bacterins against challenge with the homologous strain and heterologous challenge with 12939 (Hau et al., 2021). We found both bacterins were able to provide homologous protection; however, only HS069 was able to protect against challenge with 12939, as seen in this study (Hau et al., 2021). This absence of protection was also seen when pigs vaccinated with a Nagasaki bacterin were challenged with the serovar 2 strain Takikawa 188 (Takahashi et al., 2001). The results of the present study considered with previous work evaluating Nagasaki bacterins (Hau et al., 2021; Takahashi et al., 2001) indicate encapsulation may play a role in the reduced efficacy of Nagasaki as a bacterin in protection against heterologous challenge. The increased encapsulation of this strain may block surface protein antigens and stimulate a more capsule directed immune response, which would provide a response more likely to be serovar specific. Future work should further investigate the immune response to bacterins to better understand the response to capsule and cellular protein *in vivo*.

Here, we report the use of an acapsular mutant of *G. parasuis* strain HS069 as a formalin inactivated bacterin. We found the HS069Δcap bacterin was able to produce comparable titers to the HS069 bacterin. Additionally, vaccination with HS069Δcap was able to provide protection against the virulent parent strain and three heterologous strains. This indicates while capsular immunity may make up a portion of the response to encapsulated *G. parasuis* bacterins, it is not essential to provide protection against all *G. parasuis* strains. In contrast, protection against Nagasaki was only seen with the HS069 bacterin which could stimulate antibodies directed at capsular polysaccharide. While both bacterin vaccine groups produced significantly more antibody directed at Nagasaki than the sham vaccinated controls, the antibody was only able to provide protection when the vaccine strain produced capsular polysaccharide and antibody to that polysaccharide could be generated, which we suspect to be associated with the degree of encapsulation of Nagasaki as compared to HS069. This study confirms the importance of strain selection in the generation of bacterin vaccines, as capsule is essential for protection against some *G. parasuis* strains but in some cases appears to hinder the development of a protective immune response against heterologous strains.

CRediT authorship contribution statement

Hau Samantha: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. **Weinert Lucy:** Investigation, Writing – original draft, Writing – review & editing. **Luan Shi-Lu:** Investigation, Writing – original draft, Writing – review & editing. **Rycroft Andrew:** Investigation, Writing – original draft, Writing – review & editing. **Langford Paul:** Investigation, Writing – original draft, Writing – review & editing. **Maskell Duncan:** Investigation, Writing – original draft, Writing – review & editing. **Wren Brendan:** Investigation, Writing – original draft, Writing – review & editing. **Brockmeier Susan:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Tucker Alexander:** Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Steven Kellner and Andrew Von Weber for their brilliant technical assistance, Judith Stasko for her assistance with electron microscopy, and the animal caretakers, without whom the animal studies would not have been possible. Opinions expressed in this paper are not reflective of the policies or views of USDA or ARS. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer. Mutant generation was supported by a Longer and Larger (LoLa) grant from the Biotechnology and Biological Sciences Research Council (BBSRC grant numbers BB/G020744/1, BB/G019177/1, BB/G019274/1 and BB/G018553/1), the UK Department for Environment, Food and Rural Affairs, and Zoetis awarded to the Bacterial Respiratory Diseases of Pigs-1 Technology (BRaDP1T) consortium.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2025.110509.

References

Aragon, V., Cerda-Cuellar, M., Fraile, L., Mombarg, M., Nofrarias, M., Olvera, A., Sibila, M., Solanes, D., Segales, J., 2010. Correlation between clinico-pathological outcome and typing of Haemophilus parasuis field strains. Vet. Microbiol 142, 387–393.

Aragon, V., Segales, J., Tucker, A.W., 2019. Glasser's Disease. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W., Zhang, J. (Eds.), Diseases of Swine. John Wiley & Sons, Inc, Hoboken, New Jersey, pp. 844–853.

Bak, H., Riising, H.J., 2002. Protection of vaccinated pigs against experimental infections with homologous and heterologous Haemophilus parasuis. Vet. Rec. 151, 502–505.

Borrathybay, E., Sawada, T., Kataoka, Y., Okiyama, E., Kawamoto, E., Amao, H., 2003. Capsule thickness and amounts of a 39 kDa capsular protein of avian Pasteurella multocida type A strains correlate with their pathogenicity for chickens. Vet. Microbiol 97, 215–227.

Braucher, D.R., Henningson, J.N., Loving, C.L., Vincent, A.L., Kim, E., Steitz, J., Gambotto, A.A., Kehrli, M.E., Jr, 2012. Intranasal vaccination with replication-defective adenovirus type 5 encoding influenza virus hemagglutinin elicits protective immunity to homologous challenge and partial protection to heterologous challenge in pigs. Clin. Vaccin. Immunol. 19, 1722–1729.

Brockmeier, S.L., Loving, C.L., Mullins, M.A., Register, K.B., Nicholson, T.L., Wiseman, B. S., Baker, R.B., Kehrli, M.E., Jr, 2013. Virulence, transmission, and heterologous protection of four isolates of Haemophilus parasuis. Clin. Vaccin. Immunol. 20, 1466–1472.

Brockmeier, S.L., Register, K.B., Kuehn, J.S., Nicholson, T.L., Loving, C.L., Bayles, D.O., Shore, S.M., Phillips, G.J., 2014. Virulence and draft genome sequence overview of multiple strains of the swine pathogen Haemophilus parasuis. PLoS One 9, e103787.

Cerda-Cuellar, M., Naranjo, J.F., Verge, A., Nofrarias, M., Cortey, M., Olvera, A., Segales, J., Aragon, V., 2010. Sow vaccination modulates the colonization of piglets by Haemophilus parasuis. Vet. Microbiol 145, 315–320.

Croucher, N.J., Lochen, A., Bentley, S.D., 2018. Pneumococcal vaccines: host interactions, population dynamics, and design principles. Annu Rev. Microbiol 72, 521–549.

Eberle, K.C., Hau, S.J., Luan, S.L., Weinert, L.A., Stasko, J.A., Wang, J., Peters, S.E., Langford, P.R., Rycroft, A.N., Wren, B.W., Maskell, D.J., Tucker, A.W., Brockmeier, S.L., Consortium, B.R.T., 2020. Generation and evaluation of a Glaesserella (Haemophilus) parasuis capsular mutant. Infect. Immun.

Gasparini, R., Panatto, D., 2011. Meningococcal glycoconjugate vaccines. Hum. Vaccin 7, 170–182.

Hau, S.J., Eberle, K.C., Brockmeier, S.L., 2021. Importance of strain selection in the generation of heterologous immunity to Glaesserella (Haemophilus) parasuis. Vet. Immunol. Immunopathol. 234, 110205.

Howell, K.J., Weinert, L.A., Chaudhuri, R.R., Luan, S.L., Peters, S.E., Corander, J., Harris, D., Angen, O., Aragon, V., Bensaid, A., Williamson, S.M., Parkhill, J., Langford, P.R., Rycroft, A.N., Wren, B.W., Holden, M.T., Tucker, A.W., Maskell, D.J., Consortium, B.T., 2014. The use of genome wide association methods to investigate pathogenicity, population structure and serovar in Haemophilus parasuis. BMC Genom. 15, 1179.

Howell, K.J., Weinert, L.A., Peters, S.E., Wang, J., Hernandez-Garcia, J., Chaudhuri, R.R., Luan, S.L., Angen, O., Aragon, V., Williamson, S.M., Langford, P.R., Rycroft, A.N., Wren, B.W., Maskell, D.J., Tucker, A.W., 2017. "Pathotyping" multiplex PCR assay for Haemophilus parasuis: a tool for prediction of virulence. J. Clin. Microbiol 55, 2617–2628.

Jacques, M., Foiry, B., 1987. Electron microscopic visualization of capsular material of Pasteurella multocida types A and D labeled with polycationic ferritin. J. Bacteriol. 169, 3470–3472.

Kawamoto, E., Okiyama, E., Sasaki, H., Sawada, T., Mikazuki, K., Ueshiba, H., 2007. Ultrastructural characteristics of the external surfaces of Pasteurella pneumotropica from mice and Pasteurella multocida from rabbits. Lab Anim. 41, 285–291.

Kelly, D.F., Moxon, E.R., Pollard, A.J., 2004. Haemophilus influenzae type b conjugate vaccines. Immunology 113, 163–174.

Kielstein, P., Rapp-Gabrielson, V.J., 1992. Designation of 15 serovars of Haemophilus parasuis on the basis of immunodiffusion using heat-stable antigen extracts. J. Clin. Microbiol 30, 862–865.

Macedo, N., Gottschalk, M., Strutzberg-Minder, K., Van, C.N., Zhang, L., Zou, G., Zhou, R., Marostica, T., Clavijo, M.J., Tucker, A., Aragon, V., 2021. Molecular characterization of Glaesserella parasuis strains isolated from North America, Europe and Asia by serotyping PCR and LS-PCR. Vet. Res 52, 68.

Martin de la Fuente, A.J., Gutierrez Martin, C.B., Perez Martinez, C., Garcia Iglesias, M.J., Tejerina, F., Rodriguez Ferri, E.F., 2009. Effect of different vaccine formulations on the development of Glasser's disease induced in pigs by experimental Haemophilus parasuis infection. J. Comp. Pathol. 140, 169–176.

McCaig, W.D., Loving, C.L., Hughes, H.R., Brockmeier, S.L., 2016. Characterization and vaccine potential of outer membrane vesicles produced by Haemophilus parasuis. PLoS One 11, e0149132.

Miniats, O.P., Smart, N.L., Ewert, E., 1991a. Vaccination of gnotobiotic primary specific pathogen-free pigs against Haemophilus parasuis. Can. J. Vet. Res. 55, 33–36.

Miniats, O.P., Smart, N.L., Rosendal, S., 1991b. Cross protection among Haemophilus parasuis strains in immunized gnotobiotic pigs. Can. J. Vet. Res. 55, 37–41.

Nielsen, R., 1993. Pathogenicity and immunity studies of Haemophilus parasuis serotypes. Acta Vet. Scand. 34, 193–198.

Oliveira, S., Blackall, P.J., Pijoan, C., 2003. Characterization of the diversity of Haemophilus parasuis field isolates by use of serotyping and genotyping. Am. J. Vet. Res. 64, 435–442.

Rosenberg, M., Gutnick, D., Rosenberg, E., 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett. 9, 29–33.

Sadarangani, M., 2018. Protection against invasive infections in children caused by encapsulated bacteria. Front Immunol. 9, 2674.

Smart, N.L., Miniats, O.P., 1989. Preliminary assessment of a Haemophilus parasuis bacterin for use in specific pathogen free swine. Can. J. Vet. Res 53, 390–393.

Takahashi, K., Naga, S., Yagihashi, T., Ikehata, T., Nakano, Y., Senna, K., Maruyama, T., Murofushi, J., 2001. A cross-protection experiment in pigs vaccinated with Haemophilus parasuis serovars 2 and 5 bacterins, and evaluation of a bivalent vaccine under laboratory and field conditions. J. Vet. Med Sci. 63, 487–491.

Wang, X., Xu, X., Wu, Y., Li, L., Cao, R., Cai, X., Chen, H., 2013. Polysaccharide biosynthesis protein CapD is a novel pathogenicity-associated determinant of Haemophilus parasuis involved in serum-resistance ability. Vet. Microbiol 164, 184–189.

Zehr, E.S., Lavrov, D.V., Tabatabai, L.B., 2012. Comparison of Haemophilus parasuis reference strains and field isolates by using random amplified polymorphic DNA and protein profiles. BMC Microbiol 12, 108.

Zehr, E.S., Tabatabai, L.B., 2011. Detection of a bacteriophage gene encoding a Mu-like portal protein in Haemophilus parasuis reference strains and field isolates by nested polymerase chain reaction. J. Vet. Diagn. Invest. 23, 538–542.