Evaluation of the recombinant proteins RlpB and VacJ as a vaccine for protection against *Glaesserella parasuis* in pigs

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**Abstract**

**Background:** *Glaesserella parasuis*, the causative agent of Glässer’s disease, is widespread in swine globally resulting in significant economic losses to the swine industry. Prevention of Glässer’s disease in pigs has been plagued with an inability to design broadly protective vaccines, as many bacterin based platforms generate serovar or strain specific immunity. Subunit vaccines are of interest to provide protective immunity to multiple strains of *G. parasuis*. Selected proteins for subunit vaccination should be widespread, highly conserved, and surface exposed.

**Results:** Two candidate proteins for subunit vaccination (RlpB and VacJ) against *G. parasuis* were identified using random mutagenesis and an in vitro organ culture system. Pigs were vaccinated with recombinant RlpB and VacJ, outer membrane proteins with important contributions to cellular function and viability. Though high antibody titers to the recombinant proteins and increased interferon-γ producing cells were found in subunit vaccinated animals, the pigs were not protected from developing systemic disease.

**Conclusions:** It appears there may be insufficient RlpB and VacJ exposed on the bacterial surface for antibody to bind, preventing high RlpB and VacJ specific antibody titers from protecting animals from *G. parasuis*. Additionally, this work confirms the importance of utilizing the natural host species when assessing the efficacy of vaccine candidates.

**Keywords:** Subunit vaccine, Glässer’s disease, *Glaesserella parasuis*

**Background**

*Glaesserella parasuis* is a Gram-negative bacterial member of the *Pasteurellaceae* family and the causative agent of Glässer’s disease, which is characterized by a fibrinous polyserositis, meningitis, and arthritis. *G. parasuis* can cause high morbidity and mortality in herds resulting in significant losses to the swine industry annually [1].

There are 15 identified serovars of *G. parasuis*; however, many isolates are untypable [2]. Multiple serovars can circulate within a herd, although it appears some serovars are more capable of causing systemic disease [3, 4]. To prevent *G. parasuis* disease in the swine industry, efforts have focused on developing broadly protective vaccines. Commercially available *G. parasuis* vaccines are predominantly based on a bacterin platform. Bacterins have been shown to provide good homologous protection [5–7]; however, this protection can be serovar or strain specific [7–10], leaving swine susceptible to disease with other serovars or strains in the field. Currently,
no available vaccine is able to provide broad cross protection for *G. parasuis*. This may be due in part to the bacterial capsule, which is serovar specific and functions to mask other antigens on the bacterial surface that may contribute to the protective immune response [11, 12]. The importance of a vaccine conferring heterologous protection has led to the pursuit of alternative vaccine platforms that avoid the generation of capsule directed immunity, such as protein and peptide vaccines. Antigens targeted for *G. parasuis* protein and peptide vaccines should be highly conserved and widespread amongst isolates and found on the surface of the bacterium.

Several mechanisms have been employed to identify subunit vaccine candidates, including the use of hyper-immune or post-challenge serum from pigs to identify proteins separated by gel electrophoresis and in silico prediction methods [13–15]. In this report, we utilized a previously reported functional genomic screen to identify subunit vaccine candidates [16]. This screen identifies proteins associated with bacterial fitness and resulted in the selection of RlpB and VacJ as vaccine candidates. The *rlpB* gene (*lptE*) is best studied in *Escherichia coli*. RlpB is a low abundance outer membrane lipoprotein that functions in outer membrane assembly, specifically in mobilizing lipopolysaccharide to the outer membrane’s outer surface, and plays an essential role in cellular viability [17–19]. The *vacJ* gene has been assessed in *G. parasuis* previously [20]. VacJ is an outer membrane lipoprotein that contributes to outer membrane integrity [20]. It has also been associated with stress tolerance, serum resistance, and host cell interaction in *G. parasuis* and other Gram negative pathogens [20–23]. Additionally, the *vacJ* gene was previously assessed for potential as a subunit vaccine against *G. parasuis* in a guinea pig model of disease [15]. In order to assess antigenicity and the potential of recombinant RlpB and VacJ (rRlpB and rVacJ) to stimulate a protective immune response in swine, we vaccinated and boosted naïve pigs with rRlpB and rVacJ 3 weeks apart. Their antibody response was quantified and protection was evaluated through challenge with the *G. parasuis* strain HS069.

**Results**

**Comparison of RlpB and VacJ sequence identity**

RlpB and VacJ amino acid sequences were compared to evaluate protein sequence diversity among *G. parasuis* isolates. The genome sequence was obtained for 11 *G. parasuis* strains representing 9 different serovars and amino acid sequences of RlpB and VacJ were generated. The *rlpB* gene was obtained for 9 of the 11 strains, the SW114 and 174 genomes are both draft sequences that contain gaps and no *rlpB* was identified. The RlpB amino acid sequence for the remaining 9 strains showed an identity greater than 96% among all strains. A complete *vacJ* gene was present in 9 of the 11 strains. The *vacJ* gene was positioned near the end of a contig in MN-H and was absent from SW140, which may be associated with gaps in the genome of these strains. Amino acid identity among the other 9 strains revealed high conservation, with a 98% or higher identity between isolates.

**Antibody response to vaccination**

Antibody titers (IgG) were determined by ELISA for rRlpB and rVacJ. Minimal reactivity was seen in animals prior to vaccination. Modest increases in IgG titer to rRlpB and rVacJ were seen in the control and bacterin vaccinated groups prior to challenge, while significant increases in titer with a memory response were seen to both rRlpB and rVacJ for the subunit vaccinated pigs (Fig. 1a and b). Additionally, animals were screened for antibody response to *G. parasuis* HS069. There was an increase in titer for bacterin vaccinated animals, but no change in titer for subunit vaccinated or control animals (Fig. 1c). Titers for bacterin vaccinated animals were significantly higher at day 21 (*p =* 0.03) and day 42 (*p =* 0.01) than that of subunit vaccinated and control animals.

Western blotting was utilized to evaluate the specificity of the antibody response. Reactivity to *G. parasuis* HS069 whole cell sonicate was not seen at 25 kDa or 35 kDa, which would correlate to intact RlpB and VacJ respectively (Fig. 2a); however, some reactivity was noted at lower molecular weights. Probing with serum from the bacterin vaccinated animals revealed no reactivity to the recombinant proteins (Fig. 2b).

**Cell mediated immune response**

Peripheral blood mononuclear cells (PBMCs) were collected at the time of boost (day 21), 1 week after boost (day 28), and at challenge (day 42) to evaluate the prevalence of interferon-γ (IFN-γ) secreting cells. Animals immunized with the subunit vaccine were found to have more IFN-γ producing cells showing reactivity to pooled rRlpB and rVacJ than control animals and bacterin vaccinated animals on day 21 (*p =* 0.014 and 0.006, respectively), but differences did not reach the statistical threshold on day 28 and 42 (Fig. 3). Additionally, more IFN-γ producing PBMCs were noted in the subunit vaccine group on day 21 than day 28 or 42, although this was not statistically significant. Minimal reactivity was seen in both the control animals and subunit vaccinated animals to stimulation with heat killed *G. parasuis* HS069, significantly less than that seen in HS069 bacterin vaccinated animals on day 21 and 28 (*p =* 0.01) (Fig. 3).
Evaluation of protection from *G. parasuis* challenge

Challenge with *G. parasuis* HS069 caused severe clinical signs (neurologic signs or severe depression) which were exhibited by 5/6 control pigs leading to euthanasia on days 2–5 after challenge (Fig. 4). *G. parasuis* was isolated from at least one site [serosal surface, joint fluid, cerebrospinal fluid (CSF), bronchoalveolar lavage fluid (BALF)] in all five clinically ill control animals. One control pig survived until the end of the study period (day 12 post-challenge) and *G. parasuis* was not isolated from any site samples collected at the time of necropsy.

Four of the six subunit vaccinated pigs also exhibited severe clinical signs and were euthanized on days 2–5...
after challenge (Fig. 4). *G. parasuis* was isolated from at least one site in all four of the clinically ill animals. Two subunit vaccinated pigs exhibited no clinical signs during the study period and were culture negative for *G. parasuis* from site samples at necropsy. No difference in survival time was noted between the control animals and those vaccinated with the subunit vaccine (*P* = 0.53).

Bacterin vaccinated pigs showed significantly better protection than control or subunit vaccinated pigs (*P* < 0.01 and *P* = 0.02, respectively). All six bacterin vaccinated pigs survived until the end of the challenge period and exhibited no clinical signs of *G. parasuis* disease (Fig. 4). At necropsy, *G. parasuis* was isolated in the BALF of 3/6 pigs, but no *G. parasuis* was cultured in the serosal swab, joint fluid, or CSF of any of the bacterin vaccinated animals.

**Discussion**

*G. parasuis* vaccine development has suffered from difficulty generating a broadly cross-protective vaccine. Many platforms have been attempted: whole cell bacterins, outer-membrane vesicles, live avirulent strains, and a variety of subunit vaccines [5, 6, 13, 15, 24–27]. While homologous protection with whole cell vaccines is often high, those studies investigating heterologous protection show less efficacy [7–10]. This is thought to
be associated with the serovar or strain specific response of capsular polysaccharide and some protein antigens. Protein subunit vaccines have good potential to generate a broadly protective immune response if the antigen is widespread, highly conserved, and exposed on the surface of the bacterium or secreted. In this study, we determined two outer membrane proteins, RlpB and VacJ, as subunit vaccine candidates and assessed the capacity of these proteins to protect against G. parasuis disease in pigs.

RlpB and VacJ are outer membrane proteins with important contributions to cellular function, including membrane stability and resistance to stressors such as complement [18, 20, 21]. Here, we found RlpB and VacJ play a role in colonization of an in vitro organ culture (IVOC) of swine respiratory epithelium and are highly conserved in isolates of G. parasuis of different serovars, making them good subunit vaccine candidates. Additionally, VacJ has previously been utilized in a guinea pig model of G. parasuis disease and found to confer good protection [15]. RlpB and VacJ were expressed as recombinant proteins, purified, and used to vaccinate cesarean derived, colostrum deprived (CDCD) pigs. We found the proteins to be highly immunogenic and capable of stimulating a high antibody titer and an anamnestic antibody response following a second vaccination (Fig. 1). We also detected cellular response to the recombinant proteins through detection of IFN-γ secreting PBMCs (Fig. 3). This response was highest at 21 days post vaccination and declined after boost vaccination, consistent with previous reports of protein specific IFN-γ producing cells [16]. Although response to subunit vaccination was noted in both antibody titer and reactive PBMCs, it was unable to protect vaccinated animals from challenge with the homologous G. parasuis strain (Fig. 4).

To better understand the lack of protection seen after subunit vaccination, we evaluated the reactivity of antisera generated from subunit vaccination and bacterin vaccination. Western blotting revealed no reactivity between pooled anti-sera from subunit vaccinated pigs and whole cell sonicate at the 25 kDa and 35 kDa sizes consistent with RlpB and VacJ; however, reactivity was seen at low molecular weights, which may indicate protein degradation in the vaccine (Fig. 2a). There was also no reactivity between pooled anti-sera from bacterin vaccinated pigs and rRlpB and rVacJ (Fig. 2b). We suspect the absence of reactivity is associated with limited expression of RlpB and VacJ by G. parasuis HS069, which is consistent with previous reports in E. coli for RlpB [17], or associated with minimal exposure of these lipo-proteins at the bacterial surface. Low expression or minimal exposure of RlpB and VacJ in vivo would also prevent high antibody titers to these proteins from providing protection to challenged pigs. Additionally, the recombinant proteins used for vaccination in this study were isolated under denaturing conditions and refolded, which may have altered the presentation of some antigens.

Although this study included small animal numbers in each group (n = 6), group size was sufficient to see differences in protection and immune reactivity between bacterin vaccinated animals and subunit vaccinated animals. Additionally, the animals included in this study were crossbred with an unknown genetic background which may increase within group variation; however, this is consistent with production settings.

Conclusions
Here, we found vaccination of pigs with rRlpB and rVacJ was not capable of inducing a protective immune response, making rRlpB and rVacJ unsuitable for a subunit vaccine to combat G. parasuis disease. Further, this report confirms the importance of testing vaccine efficacy in the natural host species. Rodent models for G. parasuis do not adequately represent disease in the natural host, as rodents do not develop the typical signs of G. parasuis and challenge requires inoculation via an artificial route, typically intraperitoneal or intravenous. These factors make rodent models a starting point for screening subunit vaccine components for G. parasuis, but the translation to swine is not exact and further testing in pigs is required.

Methods
Bacterial isolates and growth conditions
Transposon mutagenesis and cloning was done with the G. parasuis serovar 5 isolate 29755, a virulent strain isolated from the lung of a pig diagnosed with polyserositis [28, 29]. G. parasuis HS069, a virulent serovar 5 isolate from the lungs of a pig with respiratory disease caused by G. parasuis [30], was utilized for bacterin production and challenge. G. parasuis was grown using brain heart infusion base (BHI) (BD Biosciences, San Jose, CA) supplemented with 10% heat-inactivated horse serum and 0.1 mg/mL nicotinamide adenine dinucleotide (NAD). Bacto agar (BD Biosciences) was added to generate solid media.

*Escherichia coli* strains were grown on Luria-Bertani (LB) agar or in LB broth (Thermo Fisher Scientific, Waltham, MA). For expression, *E. coli* was grown in 2YT broth (Life Technologies, Carlsbad, CA). Transformants were selected using 100 µg/mL kanamycin (Sigma-Aldrich, St. Louis, MO).

Selection of candidate proteins
Selection of candidate proteins was accomplished through a previously utilized protocol combining
functional genomic screening using a TraDIS library generated in *G. parasuis* 29755 and in silico bioinformatics to detect outer membrane proteins important to bacterial fitness in a swine respiratory epithelium in vitro organ culture (IVOC) system [16]. Briefly, genes of interest were defined by a loss of fitness in the IVOC system coupled with a transposon insertion in that gene. These genes were then evaluated for subcellular localization using PSORTb (http://db.psort.org/) and LocateP (http://www.cmbi.ru.nl/locatep-db/cgi-bin/locatepdb.py) databases. Localization utilizing literature mining also enabled detection of surface-associated proteins, specifically proteins known to be cell wall anchored or extracellular (lipid-anchored or secretory). Proteins with transmembrane domains in the middle of the coding sequence were excluded. Coding sequences of genes of interest were compared utilizing 11 *G. parasuis* genomes to determine cross protection potential, as previously described [16]. The screening resulted in the selection of two genes of interest, *rlpB* and *vacJ*, which had not been previously published, patented, or assessed for protection against *G. parasuis* in swine.

**Evaluation of VacJ and RlpB sequence identity**

The amino acid sequences of RlpB and VacJ were compared using Geneious 9.0.5 (Biomatters Ltd., Auckland, New Zealand). Sequences were obtained for 11 *G. parasuis* strains representing 9 serovars from National Center for Biotechnology Information (NCBI) for SH0165 (CP001321), Nagasaki (APBT00000000), SW114 (APBU00000000), MN-H (APBV00000000), 12, 939 (APBW00000000), 27, 755 (ABKM00000000), 84–15, 995 (APBX00000000), H465 (APBY00000000), D74 (ABPZ00000000), 174 (APCA00000000), and SW140 (APCB00000000). The *rlpB* and *vacJ* genes were extracted from the genomes, translated, and compared using a multiple sequence alignment.

**Cloning and production of rVacJ and rRlpB**

The DNA encoding the lipoproteins *rlpB* and *vacJ* without the putative secretion sequence (*rlpB*16-164aa and *vacJ*19–248aa) was amplified from *G. parasuis* 29755 with the primer pairs listed in Table 1. The amplified PCR products were cloned into the pET-30Ek/LIC expression vector (Millipore Sigma, Burlington, MA) according to the manufacturers’ protocols. The plasmid constructs were verified by PCR and Sanger sequencing.

The constructed expression vectors were transformed to *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) for expression. Fresh 2YT media (1–6 L) was inoculated with overnight growth of *E. coli* BL21 (DE3) and grown to an OD595 of 0.6. Expression was induced with 1 mM IPTG (Sigma-Aldrich). Protein expression was verified with SDS-PAGE from whole cell lysates.

**Proteins purification from batch cultures**

**Isolation and Ni-NTA of rRlpB**

Cell pellets containing *rRlpB* were resuspended in binding buffer (10 mM imidazole, 300 mM NaCl, 50 mM phosphate, pH 8.0) and sonicated on ice in the presence of benzonase and r-lysozyme. Lysates were centrifuged and the supernatant containing soluble rRlpB was loaded onto a Ni NTA His-bind Superflow (Novagen) column in binding buffer. The column was washed in binding buffer, followed by 20 mM imidazole, 300 mM NaCl, 50 mM phosphate pH 8.0 before rRlpB was eluted with 250 mM imidazole, 300 mM NaCl, 50 mM phosphate pH 8.0.

**Isolation and Ni-NTA of rVacJ**

Cell pellets containing *rVacJ* protein as inclusion bodies were re-suspended in BugBuster Protein Extraction Reagent (Millipore Sigma, Burlington, MA) in the presence of benzonase and r-Lysozyme. After centrifugation the pellet was re-suspended in BugBuster reagent and pelleted followed by four washes in dilute BugBuster reagent (1:10). The rVac containing pellet was dissolved in binding buffer (8 M urea, 0.1 M sodium phosphate buffer, 0.01 M Tris, pH 8.0) and loaded onto Ni NTA His-bind Superflow column (Novagen). The column was washed in binding buffer, then 8 M urea in 0.1 M sodium phosphate buffer, 0.01 M Tris, pH 6.5 before elution in 8 M urea in 0.1 M sodium phosphate buffer, 0.01 M Tris pH 4.5.

**Anion exchange of rRlpB and rVacJ under denaturing conditions using 8 M urea and dithioreitol (DTT)**

Both the *rRlpB* and *rVacJ* containing fractions from the Ni-NTA columns were identified using A280nm and SDS-PAGE and then dialysed into 30 mM Tris/HCl pH 8.5. Most of the rVac precipitated due to the removal of the urea, while the rRlpB remained in solution. Urea, DTT and further Tris were added to the dialysate to produce a solution of protein in 8 M Urea, 15 mM DTT, 30 mM Tris pH 8.5, which was incubated for 3 h at 37°C. The pH of the mixture was adjusted to pH 8.0 and 0.2 μM filtered immediately before loading it onto Source Q - FPLC. Anion exchange was performed using a 25-column volume gradient (low salt buffer: 30 mM

| Table 1 | Primers utilized to amplify vacJ and rlpB sequences |
|---|---|
| Primer | Sequence (5’-3’) |
| P1 (VacJ_ForA) | GACGAGCAAGATGTTGACTGCTACATTTGTGATCATCTGAA |
| P2 (VacJ_RevA) | GAGGAGAGCCCCTGAATTCAATTTTTTATGCTTCTTC |
| P3 (RlpB_ForA) | GACGACGACAGATGTTGGGCGTTGATTGGAATAAAAA |
| P4 (RlpB_RevA) | GAGGAGACGCCGTTTATTTGCTATTTTCTTCTTTC |
Tris pH 8.0, 8 M urea, 2 mM DTT and High salt buffer: 30 mM Tris pH 8.0, 8 M urea, 2 mM DTT, 0.5 M sodium chloride). Eluted peak samples were detected using A280nm and analysed using SDS-PAGE. Proteins were dialysed 3 times in PBS. Overnight dialysis was followed by dialysis in fresh PBS the next day, followed by another change of PBS and a second overnight dialysis. Samples were 0.2 μM filtered to remove any precipitated protein. Purified proteins were submitted to the Iowa State University Protein Facility in Ames, IA for verification by LC-MS/MS.

**Western blotting**

Western blotting was used to detect reactivity of sera from bacterin vaccinated pigs to rRlpB and rVacJ and reactivity of sera from subunit vaccinated pigs to whole cell sonicate from G. parasuis HS069. The recombinant proteins (2 μg) and G. parasuis HS069 sonicate (10 μg) were run on an SDS-PAGE gel and transferred to a PVDF membrane using the iBlot system (Invitrogen, Carlsbad, CA). Membranes were blocked with 10% milk in 1x Tris buffered saline with 0.05% Tween (TTBS) and probed with 5% milk in TTBS containing 1:100 or 1:1000 pooled serum from bacterin vaccinated or subunit vaccinated pigs, respectively. Goat anti-swine IgG conjugated with horse radish peroxidase (KPL, Gaithersburg, MD) at a 1:20,000 dilution in 5% milk overnight at room temperature with 100 μL of recombinant protein or HS069 in 100 mM carbonate-bicarbonate buffer (pH 9.6). Recombinant proteins were used at the following concentrations: rRlpB at 0.25 μg/mL and rVacJ at 0.125 μg/mL. HS069 was used to coat at a concentration of 0.5 μg protein per mL. Plates were washed three times prior to use with 1X PBS with 0.05% Tween 20 (PBST) and blocked for 2 h with 200 μL of 2% bovine serum albumin (BSA) in PBST. Plates were again washed three times with PBST and probed in duplicate with 100 μL of serial two-fold dilutions of swine antisera in 1% BSA in PBST for 2 h. Following three washes with PBST, protein specific IgG was detected using 100 μL of an overdose of sodium pentobarbital.

**Blood samples**

Blood samples were taken on day 0, 21, 28, and 42 in BD Vacutainer serum separator tubes (SST) (Becton Dickinson, Franklin Lakes, NJ). Serum was collected and frozen at –80°C until ELISAs were run. Additionally, blood was collected in BD Vacutainer cell preparation tubes (CPT) (Becton Dickinson) with sodium citrate for the isolation of PBMCs on day 21, 28, and 42. At necropsy, samples were collected and culture was performed on the following samples: nasal wash, serosal swab (pleural, pericardial, and peritoneal surfaces), joint fluid from hock or other affected joint, CSF, BALF, and serum.

**Antibody titer analysis via ELISA**

Antibody titers to rRlpB and rVacJ were determined using an indirect ELISA. Immulon-2 plates were coated overnight at room temperature with 100 μL of recombinant protein or HS069 in 100 mM carbonate-bicarbonate buffer (pH 9.6). Recombinant proteins were used at the following concentrations: rRlpB at 0.25 μg/mL and rVacJ at 0.125 μg/mL. HS069 was used to coat at a concentration of 0.5 μg protein per mL. Plates were washed three times prior to use with 1X PBS with 0.05% Tween 20 (PBST) and blocked for 2 h with 200 μL of 2% bovine serum albumin (BSA) in PBST. Plates were again washed three times with PBST and probed in duplicate with 100 μL of serial two-fold dilutions of swine antisera in 1% BSA in PBST for 2 h. Following three washes with PBST, protein specific IgG was detected using 100 μL of a 1:25,000 dilution of goat anti-swine IgG conjugated with horse-radish peroxidase (KPL) in 1% BSA in PBST. Plates were incubated for 1 h, washed three times with PBST, and 100 μL of tetramethylbenzidine (TMB) substrate (Life Technologies, Carlsbad, CA) was added to each well. Plates were incubated in the dark for 5 min and stopped with 50 μL of 2 N H₂SO₄. The optical density at 450 nm (OD₄₅₀) was measured with a correction at 655 nm (OD₆₅₅). The resulting data was modeled using GraphPad Prism (GraphPad Software, La Jolla, CA) as a nonlinear function of the log₁₀ dilution and the log (agonist)-versus-response variable slope four-parameter logistic model. Endpoints were interpolated by using two times the average OD of the gnotobiotic pig serum sample as the cutoff.
Evaluation of the cell-mediated immune response

The induction of cell-mediated immunity was evaluated following vaccination by enzyme-linked immunosorbent spot (ELISpot) assays. IFN-γ-secreting cells were enumerated after in vitro stimulation with rRlpB and rVacJ. Blood collected in CPT was used to isolate PBMCs as previously described [31]. IFN-γ ELISpot plates were seeded with 2.5 × 10^5 PBMCs per well with duplicate wells for each treatment. PBMCs were stimulated with the recombinant proteins in a total volume of 0.25 mL (0.5 μg/mL of each individual protein per well). Negative and positive control wells were treated with medium alone or pokeweed mitogen (0.5 μg/mL), respectively. Approximately 18 h after stimulation, ELISpot assays were completed following manufacturer’s recommendations (R&D Systems, Minneapolis, MN). Spots corresponding to IFN-γ-secreting cells were enumerated using an SSUV ImmunoSpot instrument and software (Cellular Technology Ltd., Shaker Heights, OH). The number of IFN-γ-secreting cells was calculated for each treatment in each group using the average of the duplicate wells for each pig.

Statistical analysis

Statistical analysis was completed using GraphPad Prism 7. Survival curves were generated by the product limit method of Kaplan and Meier and compared using the log-rank test. Log 10 antibody titers were compared using the average of the duplicated wells for each treatment. PBMCs were seeded with 2.5 × 10^5 PBMCs per well with duplicate wells for each treatment. PBMCs were stimulated with the recombinant proteins in a total volume of 0.25 mL (0.5 μg/mL of each individual protein per well). Negative and positive control wells were treated with medium alone or pokeweed mitogen (0.5 μg/mL), respectively. Approximately 18 h after stimulation, ELISpot assays were completed following manufacturer’s recommendations (R&D Systems, Minneapolis, MN). Spots corresponding to IFN-γ-secreting cells were enumerated using an SSUV ImmunoSpot instrument and software (Cellular Technology Ltd., Shaker Heights, OH). The number of IFN-γ-secreting cells was calculated for each treatment in each group using the average of the duplicate wells for each pig.

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Authors’ contributions

SH, CL, TN, SB designed and performed the animal study and immunologic assays. SL, JW, SP, DS, LW, AT contributed to the identification and production of the candidate proteins. PL, AR, BW, DM, AT contributed conception and acquisition of funding. All authors contributed to the drafting and revision of the manuscript. All authors of this manuscript have agreed for authorship, read and approved the manuscript, and given consent for submission and subsequent publication of the manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

All animal trials were approved by the National Animal Disease Center’s Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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