

Final Report

I. Project Title: Development of a new method for *Haemophilus parasuis* genotyping based on sequencing of antigen-coding genes.

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II. Scientific Abstract: Genotyping using the Enterobacterial Repetitive Element-based PCR (ERIC-PCR) has been traditionally used to characterize *H. parasuis* strains and to select isolates to be included in autogenous vaccines. One of the major limitations of this method is the lack of information provided regarding antigenicity of *H. parasuis* isolates. In order to address this limitation, we proposed the development of a new method for *H. parasuis* genotyping based on the sequencing of antigen coding genes. We selected a farm with high mortality associated with *H. parasuis* infection and obtained 50 isolates from diseased nursery pigs. Isolates were genotyped using the ERIC-PCR technique and groups of prevalent strains were identified. A representative isolate from each prevalent strain group was selected to be included in the autogenous vaccine. For the sequence-based method, whole cell protein profiles of representative strains of *H. parasuis* recovered from diseased pigs were probed using convalescent serum collected during the field outbreak by Western Blot. An immunopredominant protein was identified in all strains tested. This protein was sequenced and the predicted nucleotide sequence was used to develop a PCR to amplify and sequence the gene coding for this protein. The highly immunogenic protein was identified as an oligopeptide permease coded by the *OppA* gene of *H. parasuis*. This gene was amplified in all strains tested and sequenced. *OppA* partial sequencing (596 bp) was able to discriminate among all *H. parasuis* reference strains tested (12 serotypes). When used to type field isolates, however, it *OppA* sequencing grouped isolates with different genomic fingerprints and different serotypes. We have successfully identified an immunopredominant protein in *H. parasuis* and have developed a sequencing-based method to type field strains. Future studies will concentrate on evaluating the cross-protective immunity generated by the *OppA* protein.

Benefit of Research to Industry: *Haemophilus parasuis* continues to be one of the main causes of nursery mortality affecting the swine industry. Several control measures have been proposed, including the use of antibiotic therapy, vaccination, and planned exposure. In this study we have identified a highly immunogenic and species-specific protein in *Haemophilus parasuis* and have developed a new method for *H. parasuis* genotyping based on sequencing of an antigen-coding gene. The unique species-specific protein we have identified is also a strong candidate for the development of a *H. parasuis*-specific ELISA test and can potentially be used as a subunit vaccine to prevent disease caused by this agent. The immunodominant OppA protein of *H. parasuis* that we have identified in this study represents a major finding, and it has opened a new chapter on *H. parasuis* diagnostics and control. We plan to explore the uses of this gene and coded protein to improve management of *H. parasuis* infection and reduce mortality in affected swine populations.

III. Introduction

Field veterinarians rely heavily on the use of autogenous vaccines to prevent and control *H. parasuis* infections. Genotyping by ERIC-PCR is currently used to select *H. parasuis* strains to be included in herd-specific vaccines (Oliveira and Pijoan, 2004). This technique allows the characterization of *H. parasuis* genetic variability within a swine population and, consequently, the identification of prevalent groups of strains associated with high mortality. Representative isolates from each prevalent strain group are selected to be included in autogenous vaccines, and genotyping is later used to monitor the emergence of new strains in the herd (Oliveira et al., 2003; Oliveira and Pijoan, 2004). Although ERIC-PCR has been used for selection of vaccine strains for many years, the association between genotype and the expression of antigens by different strains of *H. parasuis* is currently unknown.

The ERIC-PCR technique was initially developed based on the identification of repetitive elements in the genome of enterobacteria such as *Escherichia coli* and *Salmonella* sp. These repetitive elements were later found to be highly conserved among different bacterial species. This technique uses primers that bind to these repetitive sequences and amplify the sections of DNA between them. As repetitive sequences are distributed throughout the bacterial genome, fragments of different sizes are produced during the polymerase chain reaction (PCR). Different strains have

different distributions of repetitive elements within their genomes, which results in the amplification of fragments of different sizes and, consequently, the production of different genome fingerprints. The ERIC-PCR, therefore, provides an anonymous characterization of the bacterial genome, meaning that the outcome of the reaction is a fingerprint based on different fragment sizes, not different sequences (Oliveira and Pijoan, 2004a; Oliveira et al., 2004). Although the ERIC-PCR provides an estimate of relatedness between different isolates, it does not predict if isolates with the same genomic fingerprint are expressing similar antigens.

In this project we developed and evaluated a sequence based method to characterize a *H. parasuis* protein recognized by the immune system of pigs that have survived the peak of mortality under field conditions. Our goal was to identify novel proteins potentially associated with protective immunity that were yet to be described for *H. parasuis*. Using western blot analysis, protein, and DNA sequencing, we provide a detailed characterization of these important antigens.

IV. Objectives

1. To develop a new method for *H. parasuis* genotyping based on sequencing of genes coding for antigens recognized by antibodies obtained from convalescent pigs (Funded by the Minnesota Pork Board)
2. To compare the efficacy of autogenous vaccines containing strains selected using the newly developed sequenced-based method with the gold standard for *H. parasuis* genotyping (ERIC-PCR) in a field trial (Funded by Newport Laboratories)

V. Material & Methods

Farm selection: A swine herd with high mortality (> 5%) due to *H. parasuis* infection was selected to participate in this study. This swine herd was located in Jackson MN and affected nurseries were used to conduct this experiment. Involvement of *H. parasuis* on nursery mortality was confirmed based on diagnostic history provided by the veterinarian in charge. Clinical signs observed in these nurseries included fever, respiratory distress, lameness, and central nervous system signs. Lesions associated with

H. parasuis systemic infection included mostly fibrinous polyserositis. Mortality was mainly observed between 5-6 weeks of age.

Selection of pigs for sampling: In each farm visit, 10 pigs were selected for necropsy per affected nursery and 10 pigs were selected for antibiotic treatment and bleeding (production of convalescent serum). Pig selection was based on clinical signs characteristics of *H. parasuis* infection (described above). Only pigs with rectal temperature higher than 105° F were sampled. Samples collected from euthanized pigs included swabs from systemic sites such as heart, brain, pleura, lung, joint, and pericardium.

Sample processing: Swabs collected were placed in Stuart medium and transported under refrigeration to the laboratory, where they were cultured onto 5% sheep blood agar plates with a nurse *Staphylococcus epidermidis* streak. All plates were incubated at 37°C in a 5% CO₂ atmosphere. Plates were checked for the presence of *H. parasuis* suspect colonies at 24 and 48 hours. Suspect colonies (satellitism to the *S. epidermidis* nurse streak) were re-isolated onto a new blood agar plate and incubated in similar conditions for 24 additional hours. DNA from pure cultures was extracted using PrepMan™ Ultra Sample Reagent following the manufacturer's instructions and tested by PCR described by Oliveira et al. (2001) to confirm the identity of the pathogen isolated.

ERIC-PCR genotyping: Genotyping of the isolates obtained during the *H. parasuis* outbreak was performed using the ERIC-PCR technique described by Oliveira et al. (2003). Prevalent strain groups were identified and a representative isolate from each strain group was selected to be included in an autogenous vaccine.

Sequencing of antigen coding genes: For the sequence-based method, the whole cell protein profiles of *H. parasuis* field isolates representative of each strain group and from 13 *H. parasuis* reference strains with known virulence were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Kawai et al., 2004). All *H. parasuis* isolates were grown under the same conditions described above. Overnight cultures were suspended in 1 ml of PBS and vortex vigorously for 1 minute. Each suspension was diluted in PBS to achieve a final concentration of 40 µg/50 µl.

50µl of this suspension was added to 50µl of sample buffer containing 950µl of Laemmli Sample Buffer (Bio Rad Laboratories, Hercules, CA) and 50µl of 2-Mercaptoethanol (Bio Rad Laboratories, Hercules, CA). The final solution was boiled for 3 minutes. Samples were loaded onto Protean Ready Precast Gel (4% stacking gel and 10% separating gel) (Jule, Inc, Milford, CT) and run at 25mA for 30 minutes and 35mA for 4 hours. The gels were stained with Coomassie Brilliant Blue R-250 Staining Solution (Bio Rad Laboratories, Hercules, CA) and then destained with destain solution Coomassie R-250 1x solution (Bio Rad Laboratories, Hercules, CA).

Western blot analysis using convalescent sera: Following SDS-PAGE analysis, proteins of *H. parasuis* field isolates and reference strains were transferred to PVDF membrane as described by Towbin et al. (1979). Sera collected from convalescent pigs were used as primary antibody at 1:50 dilution. Anti-Pig IgG (whole molecule) Peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) at 1:1000 dilution was used as secondary antibody. The membranes were stained using the Opti-4CN Substrate Kit (Bio Rad Laboratories, Hercules, CA) following the manufacturer's instructions. After staining the membranes, pictures were taken with a digital camera and the membranes were let dry completely. All membranes were stored refrigerated. Serum from 1 day-old piglets collected prior to suckling was used a negative control for non-specific binding. Western Blot using whole cell proteins extracted from bacterial pathogens commonly isolated from swine was performed for specificity. Specificity testing included the following: *Pasteurella multocida*, *Bordetella bronchiseptica*, *Actinobacillus pleuropneumoniae*, *A. idolicus*, *A. minus*, *A. suis*, *A. porcinus*, *Escherichia coli*, *Salmonella choleraesuis*, and *Streptococcus suis*.

Identification and characterization of the highly immunogenic protein: An immunodominant protein recognized by convalescent antibodies from naturally infected pigs was identified and sequenced at the U of M BioMedical Genomics Center (BMGC). The obtained protein sequence was blasted in the GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>) and the gene coding for this protein identified. The gene sequence available at the GenBank was used to design the PCR primers used for amplification and sequencing of the gene coding for the identified protein. Primers were designed using Primer3 software to amplify this gene by PCR in a final volume of 25 µl containing 1.5 U *Taq* polymerase, 200 µM dNTP and 1.2 µM/µl of each primer.

Cycling conditions were 15 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 50 °C and 1 min at 72°C, followed by a final step of 7 min at 72°C. After PCR, 5 µl aliquots of the reactions were visualized in a 2% agarose gel. Amplicons were then purified using QIAquick[®] PCR Purification Kit (250) (Qiagen Sciences, Maryland, USA) and 1 µl product was sequenced using the correspondent primers at BioMedical Genomics Center (BMGC) laboratories. Sequences obtained for *H. parasuis* reference strains and field strains were edited and aligned using the Clustal W algorithm in MEGA v.3.1 software.

VI. Results

***Haemophilus parasuis* field isolates:** Fifty *H. parasuis* isolates were obtained from 3 different nurseries at the selected farm. Three main groups of prevalent strains were identified using ERIC-PCR technique. One representative isolate from each prevalent group was selected to be included in an autogenous vaccine.

Detection of the highly immunogenic species-specific protein in *H. parasuis*: The western blot results showed multiple bands ranging from 27 kDa to 77 kDa for both sera obtained on day 0 and day 21. One single band with an estimated molecular weight of 52 kDa was clearly immunodominant and was observed in all *H. parasuis* strains tested, including the reference strains (Figure 1). Specificity testing revealed that this protein was present in 13 different *H. parasuis* reference strains (Figure 2) and 7 *H. parasuis* field strains recovered from pigs involved in the outbreak (Figure 3).

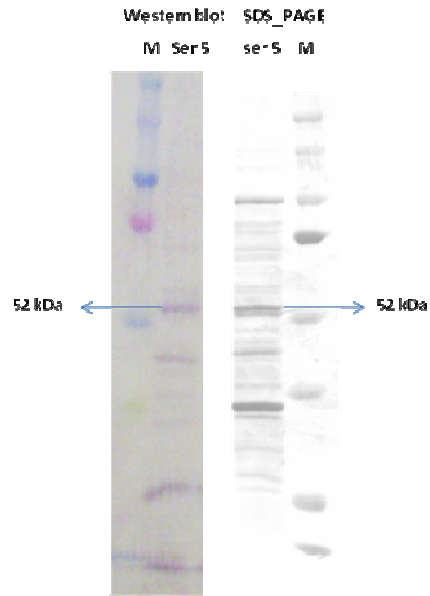


Figure 1 – 52 kDa protein identified by convalescent sera (western blot) and selected for further identification by sequencing (SDS-PAGE).

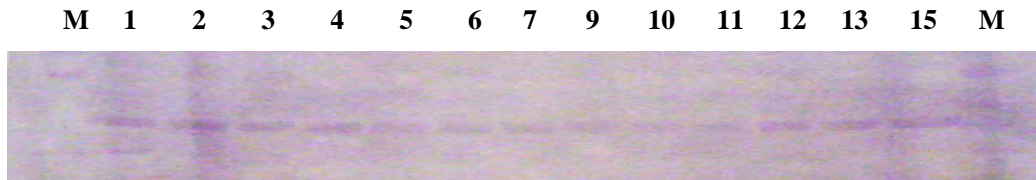


Figure 2. Detection of the immunogenic species-specific antigen in 13 *Haemophilus parasuis* reference strains by western blot. Serotypes are indicated in each well.

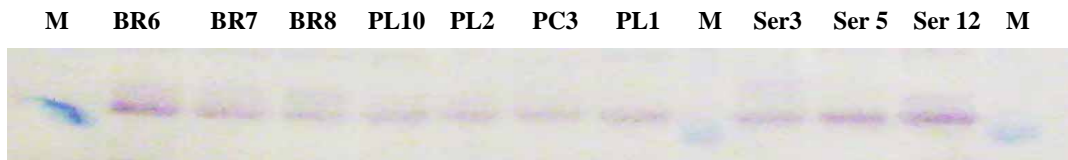


Figure 3. Detection of the immunogenic species-specific antigen in 7 *Haemophilus parasuis* field strains obtained from brain (BR), pleura (PL), and pericardium (PC) during the outbreak. *Haemophilus parasuis* references strains for serotypes 3, 5, and 12 were used as controls

Convalescent serum from 10 different pigs involved in the outbreak consistently detected the species-specific protein of *H. parasuis* during the outbreak and 3 weeks after. This protein was not recognized by the serum of piglets prior to suckling colostrums (no non-specific reaction with porcine serum) (Figure 4). Serum obtained from pigs involved in the *H. parasuis* outbreak did not recognize this protein in other bacterial pathogens commonly isolated from swine, namely *Pasteurella multocida*, *Bordetella bronchiseptica*, *Actinobacillus pleuropneumoniae*, *A. idolicus*, *A. minus*, *A. suis*, *A. porcinus*, *Escherichia coli*, *Salmonella choleraesuis* and *Streptococcus suis* (Figure 5).

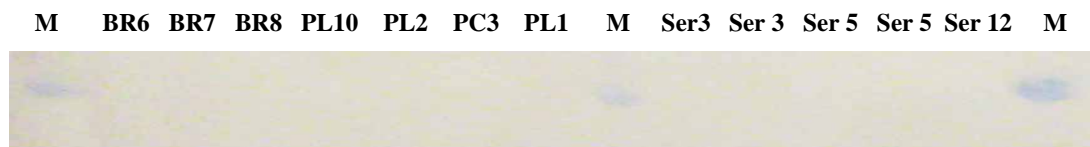


Figure 4. Specificity testing using serum from a piglet obtained prior to colostrum suckling. No non-specific detections by western blot were observed.



Figure 5. Specificity testing by western blot using serum from a convalescent pig that survived a *H. parasuis* outbreak. The highly immunogenic species-specific *Haemophilus parasuis* protein was not detected in *Actinobacillus porcinus* (AP), *Actinobacillus indolicus* (AI), *Actinobacillus minor* (AM), *Actinobacillus pleuropneumoniae* (APP), *Actinobacillus suis* (AS), *Streptococcus suis* (SS), *Bordetella bronchiseptica* (BB), *Pasteurella multocida* (PM), *Escherichia coli* (EC), *Salmonella choleraesuis* (SC). The protein was detected in *Haemophilus parasuis* strains isolated from brain (BR7) and in the reference strain for serotype 5 (Ser 5).

Characterization of the highly immunogenic and species-specific protein: The 52 kDa protein was sequenced in all outbreak strains and reference strains for serotypes 3 and 5 and was consistently identified as an oligopeptide permease A (OppA) in the GenBank. The OppA gene codes for this protein, which is a transmembrane protein responsible for transporting and internalizing 3-5 amino acids molecules in bacteria (Higgins and Hardie, 1983).

Amplification and sequencing of the OppA gene: The PCR developed to amplify the OppA gene of *H. parasuis* detected this gene in all reference and field strains tested.

Partial sequencing (596 bp) of the OppA gene amplified from *H. parasuis* reference strains allowed the differentiation of all serotypes tested (100% differentiation, Figure 6). Although OppA sequencing provided promising results when tested using reference strains, it did not differentiate among field strains with different genomic fingerprints and serotypes (Figure 7).

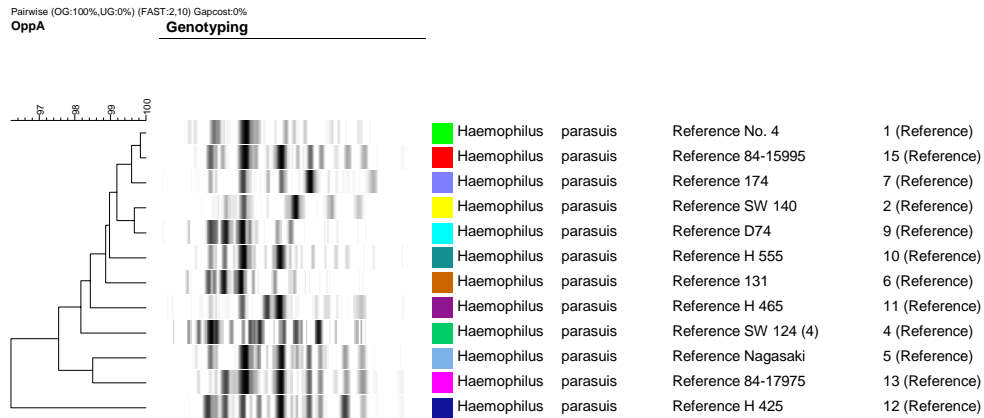


Figure 6 – Dendrogram analysis based on OppA partial sequence (596 bp) generated for *Haemophilus parasuis* strains reference strains (12 serotypes). OppA sequencing was able to differentiate all *H. parasuis* reference strains tested.

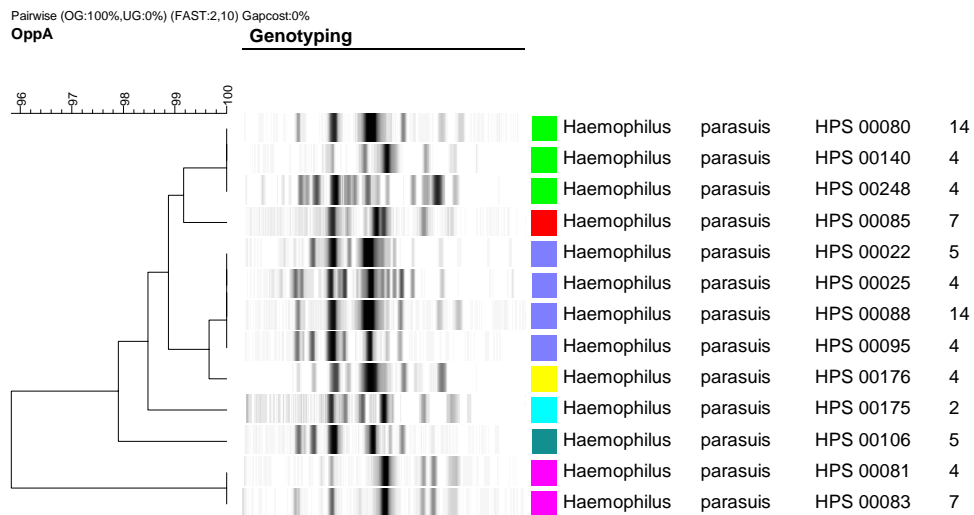


Figure 7. Dendrogram analysis based on OppA partial sequence (596 bp) generated for *Haemophilus parasuis* field strains. Straight lines and colors indicate identical OppA sequences. Isolates with different genomic fingerprints and serotypes (last column) shared similar OppA protein sequences.

Summary and relevance for the swine industry

The main objective of this project was to identify a gene coding for an immunologically relevant protein in *H. parasuis* that could be used for strain typing and selection of strains to be used in vaccines. The OppA gene identified in this project codes for an immunodominant protein recognized by the convalescent sera obtained from pigs that have survived a *H. parasuis* outbreak. To our surprise, this immunodominant protein was also highly specific for *H. parasuis*. We have not only accomplished our main goal of developing a sequence-based method for *H. parasuis* typing targeting a relevant antigen-coding gene, but we have also identified a great candidate for the development of a *H. parasuis*-specific ELISA test and a potential subunit vaccine. Our future studies will concentrate on developing a species-specific serological test to characterize antibody profiles against *H. parasuis* and evaluate the protective immunity generated by the OppA protein against different *H. parasuis* serotypes.

References

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