Streptococcus suis intranasal challenge model and identification of a potential anti-phagocytic virulence factor in S. suis supernatant

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Streptococcus suis intranasal challenge model and identification of a potential anti-phagocytic virulence factor in S. suis supernatant

by

Gayle Blair Brown

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CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation was compiled using the alternate format. Included in this dissertation is a general introduction of the dissertation and introduction to the research problem, a review of the literature regarding virulence factors of streptococci, two manuscripts concerning S. suis, and a final chapter with general conclusions that summarizes the results of the research and discusses their contribution to the understanding of S. suis-induced disease. The two manuscripts will be submitted for publication. The references cited for each chapter are found immediately following that chapter.

Statement of the Problem and Research Summary

*Streptococcus suis* (S. suis) commonly colonizes the tonsils of healthy swine. However, *S. suis* infection may lead septicemia resulting in meningitis, arthritis, septicemia, and/or pneumonia. Medicated early weaning and other similar management practices have been unable to eliminate this disease. One of the reasons these practices are unable to control *S. suis*-induced disease is that the tonsils of neonatal pigs can be colonized by *S. suis* through contact with the sow at birth or shortly thereafter. This disease remains a cause of morbidity and
mortality in herds that otherwise have a high health status.\textsuperscript{1} Currently available vaccines have limited ability to control the clinical syndrome.\textsuperscript{16}

Phagocytes play a key role in defense against systemic bacterial infection and therefore should also be central in the defense against \textit{S. suis}-induced disease. Outbreaks of \textit{S. suis}-induced disease are often associated with the occurrence of stressors (such as weaning, mixing, vaccination, PRRSV infection, or other infectious agents) which may suppress phagocyte functions.\textsuperscript{4} It has been reported that pigs treated with interleukin 1 had increased activation of neutrophils and increased resistance to \textit{S. suis} challenge.\textsuperscript{17} These observations indicate that neutrophils play a key role in controlling \textit{S. suis} infection.

In order to overcome the phagocytic defense system, a pathogen must have virulence factors to enable it to evade ingestion and/or killing by phagocytic cells.\textsuperscript{23} Several potential virulence factors of \textit{S. suis} have been identified. These include capsule, suilysin (a hemolysin), hemagglutinin, muramidase released protein and extracellular factor.\textsuperscript{5,7,8,10,12,20-22} The capsule is the only virulence factor that has been defined as critical, the other factors do not consistently correlate with virulence and are not essential for virulence.\textsuperscript{5,9} There is some correlation with capsular thickness and resistance to phagocytic killing; however, there is a report of an isolate that did not have a thick capsule but was resistant to phagocytic killing.\textsuperscript{13} This suggests that there are other as yet undescribed virulence factors associated with \textit{S. suis} that interfere with phagocyte function.

Investigation of \textit{S. suis} virulence and evaluation of \textit{S. suis} vaccines requires a challenge model which mimics the natural route of infection and reproduces the
disease syndrome. There are apparently no publications describing *S. suis* challenge models that meet this criteria. The published challenge models are intravenous inoculation of pigs, intranasal inoculation of pigs infected with other pathogens, or intravenous inoculation of mice as a model for pigs.\(^3,6,11,18\) Although these models have contributed much to the understanding of *S. suis* disease, they also limit and confound some of the information needed to better understand *S. suis* disease in swine. In addition, it has recently been reported that the mouse is probably not a good model for evaluating virulence factors for *S. suis*.\(^19\)

The objectives of the research presented in this dissertation were to develop a challenge model that would mimic the natural route of exposure in pigs without the confounding factor of other infectious agents and to better understand *S. suis* interaction with neutrophils. The first manuscript describes a challenge model that fulfilled our objectives for a reproducible model that mimicked the clinical disease observed in the field. This model has been used in two subsequent studies that resulted in papers submitted for publication. The second manuscript describes a factor that is found in the supernatant of *S. suis* incubated in saline for two hours that decreases pig neutrophil function. The results are summarized and discussed in the general conclusions.

**References**


CHAPTER 2. LITERATURE REVIEW: VIRULENCE FACTORS OF STREPTOCOCCI

General Introduction

Streptococci were first described in the 1800s as chaining coccii. Some common features of streptococci are that they are gram positive, grow in chains or pairs, and are facultative anaerobes. There are more than 40 types of streptococci as identified by a variety of grouping systems. The hemolysis pattern was one of the first ways used to categorize streptococci. Subsequently, in the 1930s, Rebecca Lancefield developed a new system for grouping streptococci. The Lancefield groupings are based on cell wall carbohydrates (group specific antigens), e.g. teichoic acids. Lancefield groups include A through H and K through V. More recently the streptococci have been divided into groups based on differences in 16S rRNA and 16S rDNA. The major groups based on 16S rDNA are pyogenic, mitis, salivarius, bovis, anginosus, and mutans. However, one grouping system is not adequate for describing all members of the genus. For example, *S. pyogenes* is a Lancefield group A streptococcus, and *S. agalactiae* is a Lancefield group B streptococcus, and in the 16S rDNA grouping system they are both in the pyogenic group. *S. pneumoniae* does not have a Lancefield grouping but is in the mitis group based on the 16S rDNA. *S. suis* is a Lancefield group D, however in the 16S rDNA system it remains ungrouped.
Streptococci are ubiquitous and can exist as commensals in their host or can cause fatal disease. Some streptococci are species specific and some cause disease in many different species. In general, the streptococci are very successful pathogens. They have many ways of surviving and evading the host immune system. Some mechanisms of evasion are unique to a certain type of streptococci, while other mechanisms are common to many types of streptococci and to many types of bacteria.

One of the major mechanisms of defense against streptococci is the native immune system, including serum proteins and phagocytic cells. Many virulence factors of streptococci interact or influence phagocyte function. This chapter will review the virulence factors of some of the major streptococcal species that induce disease in humans and animals, and, where applicable, the important interactions with phagocytic cells. The major streptococci that will be discussed are Group A *streptococci* (GAS), Group B *streptococci* (GBS), *S. pneumoniae* and *S. suis*.

**Virulence Factors Associated with the Capsule of Streptococci**

The capsules of streptococci are made of various polysaccharides. In general, the capsules are large molecular weight molecules made of 2-8 different repeating units. Biosynthesis and transport of capsule in gram positives is poorly understood. The capsular components are assembled in the cytoplasm and
transported most likely via ATP-binding cassettes to the outside of the cell. They are attached to the bacterial cell by covalent linkages.\textsuperscript{53,72}

Bacterial capsules can have many important functions. The capsule aids in survival by protecting the cell from drying, since capsules are approximately 95% water.\textsuperscript{53} In addition to preventing dehydration, the capsule contributes to transmission and adherence to host cells. Some capsular molecules bind to host receptors and initiate the first step for successful infection. Another major contribution of the capsule to virulence in the host is evasion of host immune defenses. The capsule can be antiphagocytic because of its hydrophilicity and effects on complement activation and opsonization. The capsule is non-protein and often contains molecules that are not foreign to the host, e.g. hyaluronic acid and sialic acid, making it poorly immunogenic. Different species of streptococci have different features about their capsules that are important for the success of the pathogen in the host.

**Group A Streptococcus**

The capsule of GAS is composed of hyaluronic acid. The hyaluronic acid that is found in the bacterial capsule is identical to hyaluronic acid that is found in connective tissue of animals. The hyaluronic acid capsule is encoded by the has gene cluster. This gene cluster is found in all strains of GAS and seems to be invariant.\textsuperscript{72,73} The importance of the capsule for virulence has been demonstrated using transposon-induced mutants. The LD\textsubscript{50} in mice of unencapsulated mutants was 150 times that of the parent.\textsuperscript{72,74} The unencapsulated mutants had decreased ability to colonize oral/pharyngeal epithelium of mice and decreased ability to
establish an infection in skin and soft tissues. The hyaluronic acid capsule has two major contributions to virulence, evasion of the immune system and adherence to the host cells. It evades the immune response because it is not recognized as foreign, does not contain protein, and is poorly immunogenic. Encapsulated GAS are resistant to phagocytosis by neutrophils while unencapsulated mutants are readily phagocytosed.\textsuperscript{73,74} The mechanism for the resistance is not completely understood. It was observed that unencapsulated isolates were susceptible to killing by polymorphonuclear cells (PMNs) in the presence of serum while the encapsulated isolates were more resistant. Therefore, it was thought that the capsule somehow interfered with opsonization by the complement system, making the encapsulated isolates resistant to phagocytosis. Further, experiments demonstrated that C3b bound equally to encapsulated and unencapsulated isolates. It was then proposed that the C3b bound to the capsule in a position in which it could not bind to the complement receptor on the phagocytic cell leading to resistance to phagocytosis.\textsuperscript{12,72}

The capsule of GAS is also important in adherence to host cells. CD44 is the hyaluronic acid receptor in the host and is found on epithelial cells, mesenchymal cells, and hematopoietic cells. The role of hyaluronic acid in adherence of the bacteria to epithelial cells was demonstrated using CD44 monoclonal antibodies. Antibody binding to CD44 caused a reduction of GAS binding to epithelial cells. In addition, transfection of CD44 negative cells with CD44 increased GAS binding to the CD44 transfected cells.\textsuperscript{59} It is interesting that CD44 is increased on host cells during inflammation, injury, and healing. These are conditions when GAS infections
are often seen. The increase in CD44 receptors may contribute to increased GAS binding and infection. It is clear that the hyaluronic capsule of GAS is important in virulence.

**Group B Streptococcus**

There are 9 antigenic types of GBS capsules. The most common capsule types isolated from diseased hosts are types II and III. Like most bacterial capsules, the GBS capsules are high molecular weight polymers of repeating units of 4-7 monosaccharides. The different GBS capsule types are composed of the same sugars, there are different antigenic types because these sugars are linked together differently. The different capsule types are correlated with disease in different aged hosts. For example, type III is almost always the serotype isolated from neonates and type II is the serotype isolated from adults. The reason for the different tropisms is unknown.

The capsule is an essential virulence factor for GBS. This has been demonstrated with transposon-induced mutants and antibody protection studies. The LD$_{50}$ in rats of unencapsulated mutants was 100 times that of the wild type parent. All GBS capsules have side chains containing a terminal sialic acid. The terminal sialic acid negative mutants had an LD$_{50}$ similar to the unencapsulated transposon mutant. This suggested that sialic acid was the important component of the capsule that was contributing to virulence. Sialic acid probably contributes to virulence by its interaction with the alternative pathway of complement. Sialic acid increases the affinity of Factor H for C3b (either soluble or membrane bound). When Factor H binds to C3b then Factor I breaks down C3b.
Therefore, a bacterial capsule that contains sialic acid will have less complement activation by the alternative pathway and less C3b opsonization making it more resistant to phagocytosis. The opposite effect is also true, when sialic acid is removed from GBS capsule an increase in complement activation can be measured.\textsuperscript{13,40}

\textit{S. pneumoniae}

The difference in virulence of encapsulated and unencapsulated \textit{S. pneumoniae} was first demonstrated by Griffith in 1928.\textsuperscript{25} The encapsulated isolates were much more virulent than unencapsulated isolates. The \textit{S. pneumoniae} capsule is composed of many types of sugars and ninety different antigenic types of capsule have been identified.

The importance of the capsule type in virulence has been demonstrated by transformation studies. Transformation of \textit{S. pneumoniae} cells with the capacity to encode for other capsule types has been demonstrated.\textsuperscript{22} In general, a strain can only synthesize one capsule type as determined by a specific genetic locus. However, there are regions of DNA homology in the \textit{S. pneumoniae} genome representing different serotypes. During transformation homologous recombination promotes exchange between the donor DNA and the chromosome of the recipient, resulting in bacteria of the same serotype as the donor.\textsuperscript{22} This transforming capability of \textit{S. pneumoniae} was used to demonstrate the role of capsule type in virulence. Three different isolates were used representing different capsular types and virulence characteristics in mice. The isolates were transformed with DNA encoding type 3 capsule and then the change in virulence examined. The change in
capsule type resulted in a change in virulence of the recipient. The virulence of the recipient was similar to that of the donor. The change in the virulence, however, could not be totally explained by the capsule type alone. It was therefore concluded that the capsule type contributes to virulence but other factors also contribute.\textsuperscript{32} The mechanism for one capsule type being more virulent than another capsule type remains unknown.

\textit{S. suis}

The capsule of \textit{S. suis} is composed of approximately 5 different sugars.\textsuperscript{9,15} Thirty-five serotypes have been identified based on the capsule and probably many more not yet identified.\textsuperscript{27}

It has been demonstrated that capsular thickness increases during in vivo growth and the thickness of capsule influences resistance to killing by phagocytes.\textsuperscript{51} Experiments comparing phagocytosis of encapsulated and unencapsulated isolates demonstrated that the encapsulated bacteria are poorly phagocytosed compared to unencapsulated. An unencapsulated transposon-induced mutant had an LD\textsubscript{50} of greater than 100 times that of the parent bacteria when inoculated intravenously into a pig.\textsuperscript{8} This result convincingly demonstrated the importance of the capsule in virulence, but the mechanism remains unclear. Sialic acid is one of the sugars found in the capsule of \textit{S. suis}; however, it does not appear to be important in virulence.\textsuperscript{10} Virulent isolates of \textit{S. suis} treated with enzymes to remove sialic acid remained virulent. One explanation for the difference between this and GBS, where sialic acid is key to virulence, is that the concentration of sialic acid in the \textit{S. suis} capsule is very low compared to GBS.\textsuperscript{10,15} Another explanation proposed is that
complement does not play an important role in the phagocytosis of *S. suis*; therefore, sialic acid would not contribute to inhibition of phagocytosis.\(^ {10} \) It has been demonstrated that virulent and avirulent isolates of *S. suis* are encapsulated and are equally phagocytosed. The difference between the two is that the virulent isolates survive longer in the phagocyte.\(^ {7} \) Therefore, certain capsule types may contribute to survival in phagocytes. More research is needed to identify the mechanism of the capsule in virulence.

**Virulence Factors Associated with the Cell Wall of Streptococci**

The cell walls of streptococci are made up of peptidoglycan and group specific polysaccharides (teichoic acids) and are the basis for the serologic group specific typing (Lancefield) for streptococci. The peptidoglycan is a polymer of \( \beta 1,4 \) linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) with peptide side chains attached to NAM. The common peptide side chain is L-alanine, D-glutamic acid, L-lysine and D-alanine. The peptide side chains are then cross-linked by peptide bridges.\(^ {58} \) The cross-linking of the peptides is what makes the peptidoglycan insoluble. The NAG-NAM is invariant between the streptococci, but the peptide side chains and cross-linking peptide bridges do vary between and within species. In addition to NAG-NAM and the linking peptides, the cell wall contains teichoic acids (or group specific polysaccharides) and lipoteichoic acids. Lipoteichoic acids are teichoic acids linked to the lipid portion of the cell membrane.
Typically, teichoic acids are composed of a sugar backbone, e.g., glycerol or ribitol and D-alanine. Assembly of the cell wall occurs by the transport of NAG-NAM disaccharide with its linked peptide side chain from the cytoplasm across the membrane to the surface. Once across the membrane it becomes part of the growing peptidoglycan chain. A basic function of the cell wall is to allow the bacteria to maintain shape under a range of osmotic conditions. In addition, the cell wall generates many biological responses in the host. Some of the biological responses are complement activation, leukocyte chemotaxis, cytotoxicity of host cells, and induction of IL1. The contribution to virulence of these responses is not completely clear. It seems that some of the responses contribute to virulence while others are part of the protective response of the host.

**Group A Streptococcus**

The cell wall of GAS is composed of NAG-NAM and the usual peptide side chains. The interpeptide linkages in GAS are L-alanine oligopeptides. The Group A specific polysaccharide is composed of a repeating unit backbone of rhamnose and NAG. When the peptidoglycan and polysaccharide of GAS are purified and injected into a mouse the clinical signs of chronic inflammation are observed. The chronicity of the inflammation is thought to result from the persistance of the peptidoglycan and polysaccharide. Peptidoglycan and polysaccharide of GAS are not easily broken down by phagocytic cells or enzymes in the tissues and can persist in the host up to 40 days. Fixation of complement, C5a recruitment and activation of PMNs cause inflammation. Another effect of the peptidoglycan and
polysaccharide is observed when neutrophils in serum are incubated with peptidoglycan and polysaccharide. The result is decreased ability of the neutrophils to phagocytose other bacteria. Addition of serum to the reaction mixture was required to observe the decreased phagocytic ability. One explanation given for this was that the peptidoglycan and polysaccharide activated the complement cascade and depleted the serum of complement components. The bacteria could not be opsonized and this resulted in decreased phagocytosis. Another explanation was that the peptidoglycan and polysaccharide bound the C3b and then competed with the opsonized bacteria for C3b receptors on the neutrophils resulting in decreased phagocytosis of the opsonized bacteria. The second explanation was the one thought to be most likely. The proposed mechanism of the GAS cell wall in virulence in vivo then is interference of the neutrophils ability to phagocytose bacteria. 

**Group B Streptococcus**

The GBS cell wall is composed of NAG-NAM and a group specific antigen of D-glucosamine, D-galactose, D-glucitol, and D-rhamnose. The interpeptide bridge of GBS has L-serine in place of some of the L-alanines of GAS. There is little other information regarding GBS cell wall and its interactions with the host.

**S. pneumoniae**

The cell wall of *S. pneumoniae* has been studied extensively because of its contribution to the severe clinical disease observed during *S. pneumoniae* infections. *S. pneumoniae* cell wall is composed of peptidoglycan and teichoic acid. Many peptide subunits and cross-linkages have been described. Teichoic acid of *S. pneumoniae* is unusual in that it contains choline in addition to more common
galactosamine and ribitol. Purified cell wall of S. pneumoniae is able to induce inflammation of the meninges in rabbits that is identical to that seen in pneumococcal meningitis. In contrast, the capsule induces little inflammation when it is purified and inoculated into a host. There is a long list of biological activities associated with the S. pneumoniae cell wall. The biological activities include: complement fixation, binding CD14, binding platelet activating factor receptor, induction of procoagulant activity on endothelial cells, activation of production of IL1, TNF, and IL12 from endothelium, epithelium, and leukocytes, chemotactic attraction of leukocytes, induction of NFkB, changes in the blood brain barrier permeability, cytotoxicity for choroid plexus cells and neurons.

The choline in S. pneumoniae teichoic acid binds to platelet activating factor (PAF) receptor. Binding of the pneumococci to these receptors is followed by invasion of the cell. It is also interesting that the inflammatory response induced by the cell wall results in upregulation of PAF receptors on the cells. Therefore the cell wall is considered a virulence factor in that it contributes to adherence of the bacteria.

The choline in the cell wall is also important for the activity of S. pneumoniae autolysin. The autolysin will be discussed in the protein section of this chapter, but at a certain stage of growth the S. pneumoniae produce autolysin which cleaves the NAM-L-alanine bond of the cell wall. The autolysis of S. pneumoniae cells allows release of intracellular virulence factors.

In summary, the cell wall of S. pneumoniae is important for survival of the bacteria, adhesion, and is responsible for many of the clinical signs seen in infected
individuals. Some of the responses obviously contribute to the bacteria's success while others seem not to be to the bacteria's advantage.

**S. suis**

The group D cell wall specific antigen of *S. suis* is glucosyl glycerophosphate. This type of cell wall does not persist like the cell wall of GAS, and it does not induce the chronic inflammation seen with GAS. There is no other information available on the composition or contribution to virulence of the cell wall of *S. suis.*

**Protein Virulence Factors of Streptococci**

Bacterial proteins are manufactured in the cytoplasm and then either remain there or are exported. Most proteins that remain in the cytoplasm are involved in cellular metabolism and transportation of cellular products. Exported proteins include proteins that are associated with the cell surface or released to the extracellular space. Exported proteins vary widely in their functions and the functions of many remain unknown.

The mechanisms for exporting bacterial proteins have been divided into three general types, type I or the ATP binding cassette (ABC) pathway, type II or the general secretory pathway (GSP), and type III or the contact secretion pathway. All three types of secretion pathways use ATP as an energy source. Some of the proteins involved in transportation participate in more than one of the pathways. Both Gram positive and Gram negative bacteria use the GSP and ABC
pathways, but only Gram negative bacteria have been found to use the contact secretion pathway.

The ABC pathway involves a complex of conserved proteins that transport the protein for export across the membrane. This process is not dependent on secretory proteins and results in transport of the whole protein across the membrane in one step.18

The GSP pathway is currently thought to be the pathway most commonly used. This pathway is dependent on secretory proteins, recognition of a signal sequence and translocation to the cell membrane. The signal sequence is cleaved off of those proteins that are to be released extracellularly and not cleaved off those proteins that are to remain anchored to the cell membrane.

Type III, contact secretion, has many similarities with the ABC pathway. The differences are that the type III pathway utilizes many more proteins (e.g., chaperones) and this pathway is triggered when the pathogen contacts a host cell. In addition, the production of some of the proteins to be exported is triggered when the contact secretion pathway is triggered. A defect in the pathway results in those proteins not being produced.18,49

Proteins contributing to virulence of streptococci are found in the cytoplasm, anchored to the bacterial surface, and extracellularly. The mechanism of export of each of the proteins has not yet been identified. However, this is a growing area of investigation, and it is proposed that new drugs will be developed that will target certain steps in the exportation pathways.
This section will present current information available regarding virulence proteins of streptococci. For some proteins, information about genetics, structure, and contribution to virulence is known; for other proteins, it is only known that they are produced by virulent isolates.

**Group A Streptococcus**

Many of the major protein virulence factors for GAS are under the regulation of one regulator called multiple gene regulator (mga). The gene products known to be under the control of this regulator are M protein, C5a peptidase, IgG binding protein, IgA binding protein, and serum opacity factor. Other gene products that may be under Mga control include cysteine protease (speB exotoxin) streptococcin A and oligopeptide permease. The Mga activity is sensitive to pH, temperature, CO₂ level, nutrient composition, and perhaps other factors. It is proposed that mga is a two component regulatory system; however, no sensor has been identified. Other proteins involved in virulence and produced by GAS are histone-like protein A (hpaA), enzymes (e.g., DNAases, hyaluronidase, streptokinase, NADase, proteinase, amylase and esterase), hemolysins (streptolysin O and streptolysin S) and other exotoxins, speA, speC, and speF.

A major virulence protein for GAS is M protein. The M protein is a dimeric alpha helical surface protein. The carboxy terminal is located in the membrane and the amino terminal is extracellular. The carboxy terminal is highly conserved and the amino terminal is variable. There are over 100 serotypes of M protein identified. Antibody to M protein is protective against homologous challenge, but there is little protection against a heterologous challenge. Typically, a GAS cell will have only
one type of M protein. Some M proteins are associated with induction of post-
streptococcal rheumatic fever and some are not.\textsuperscript{71} M protein is antiphagocytic and, therefore, a virulence factor. The M protein binds fibrinogen and complement inhibitor, Factor H. This prevents opsonization by complement and activation of the complement pathway. It is thought that M protein may have antiphagocytic activity in addition to inhibition of the complement system. In the absence of serum, phagocytosis was still inhibited. The mechanism for this inhibition of phagocytosis has not been identified.\textsuperscript{55,71}

The IgG binding protein and IgA binding protein contribute to virulence. These proteins are common to many streptococci. They bind antibody via the antibody Fc receptor and camouflage the bacteria.\textsuperscript{47,52}

C5a peptidase is indirectly an antiphagocytic virulence factor since it destroys C5a, which is a chemoattractant of neutrophils. By decreasing the number of neutrophils responding to the infection, the bacteria have more time to replicate and establish an infection.\textsuperscript{71}

The role of serum opacity factor is unknown, but it is associated with virulent strains and is regulated by \textit{mga}.\textsuperscript{55}

Three or four exotoxins are produced by GAS, speA, B, C, and F.\textsuperscript{55,71,76} The exotoxin, speB is a cysteine protease. It has been shown by deletion mutants to be important in virulence.\textsuperscript{29,38} Immunization of mice with purified speB protected mice against subsequent challenge. However, it’s exact role in virulence is unknown. Cysteine proteases have many activities including cleavage of fibronectin and vitronectin,\textsuperscript{31} and induction of IL1\textbeta production.\textsuperscript{30} These activities probably contribute
to invasion into the tissues. Spe B has also been reported to decrease the phagocytic activity of monocytes like cells and also induce apoptosis in that same cell line.\textsuperscript{33} Therefore, the Spe B may contribute to immune evasion. SpeA, C and F act as superantigens causing increased T cell activation and cytokine release and potentially life threatening clinical disease, other functions for these exotoxins have not been identified.\textsuperscript{55,71,76}

The two hemolysins produced by GAS are active under different conditions. Streptolysin O is an oxygen labile, thiol-activated hemolysin which is released extracellularly. This hemolysin binds to cholesterol and causes lysis of red blood cells and neutrophils. Streptolysin S is a serum and oxygen stable cytotoxin. This toxin is found extracellularly and uses an oligonucleotide as a carrier. The oligonucleotide is required for the toxin to have hemolytic activity.\textsuperscript{66} Streptolysin S causes lysis by binding to phospholipids of cell membranes and destabilizes them.\textsuperscript{71}

Finally, the HlpA histone-like protein has recently been described as a virulence factor for GAS. Besides binding to DNA in the cells, HlpA is released extracellularly and binds to heparin sulfate proteoglycans on many of the tissues. This protein may play a role in post-streptococcal disease causing glomerulonephritis.\textsuperscript{64}

Various enzymes are produced by GAS. These enzymes most likely help the bacteria to invade and damage surrounding tissues, increasing the nutrients available to the bacteria. These enzymes include nucleases, hyaluronidase, streptokinase, NADase, proteinase, amylase and esterase.\textsuperscript{55,71,76}
**Group B Streptococcus**

GBS have fewer proteins known to be associated with virulence than the GAS. The proteins that have been identified are considered to provide some virulence advantage to the GBS; however, their contribution is secondary to the major virulence factor, capsule.

The major surface proteins are α antigen, β antigen, R protein and Rib protein. The β antigen is an IgA binding protein. No function has been identified for the other proteins. These proteins are thought to contribute to virulence because they are found on virulent isolates and antibodies to them are protective against challenge in mice. These proteins are not all found in all types of GBS. The α antigen and β antigen are found in types I and II and only in a lower percentage of the type III isolates. The Rib protein is expressed on most of type III isolates and only on a low percentage of other isolate types. R protein is found in a high percentage of type II and III clinical isolates.

GBS are β hemolytic and produce an intracellular protein with hemolytic activity. A hemolysis negative mutant was less virulent following intrathoracic inoculation into an animal compared to the hemolytic parent. A positive correlation has been made between the amount of hemolysin produced and the amount of lung damage, and the hemolytic activity is inhibited by phospholipids found in surfactant in the lung. It has been proposed that the hemolysin may have a role in invasion; however, this has not been investigated.

A C5a protease, like the one found in GAS, is also produced by GBS. Using C5 deficient mice and C5 competent mice, Bohnsack, et al., demonstrated that C5a
was important in recruitment of PMNs to sites of GBS infection and that strains that produced C5ase attracted fewer polymorphonuclear cells (PMNs) to sites of GBS infection. It was proposed that the decrease in PMN recruitment allowed the bacteria extra time to multiply and spread before other mechanisms of recruitment could attract PMNs to the site.\(^6\)

Hyaluronidase, DNAase and neuraminidase are produced by some isolates.\(^71,76\) Based on research from other bacteria that produce these enzymes, they may contribute to GBS virulence.

*S. pneumoniae*

Several proteins produced by *S. pneumoniae* have been identified as virulence factors. Like the other streptococci, *S. pneumoniae* produces several extracellular enzymes. *S. pneumoniae* produces IgA1 protease which may help it evade mucosal immune defenses as well as humoral defenses since this protease cleaves sIgA, IgG, and IgM. It produces neuraminidase, which may aid in the attachment of the bacteria to mucosal epithelium, hyaluronidase, which may aid in tissue destruction and neutrophil elastase inhibitor, which may aid in evasion of nonspecific immune system.\(^2,71\)

*S. pneumoniae* produces at least three different proteins that interact with the complement system. Two of the proteins are produced early in an infection and inhibit the complement system. One of the proteins is a Factor H binding protein and the other is a C3 degrading enzyme.\(^2\) The third protein that interacts with complement is pneumolysin. Pneumolysin is a thiol-activated hemolysin, similar to streptolysin O, and is found intracellularly. The pneumolysin is considered a
virulence factor based on antibody protection studies and pneumolysin negative
mutant mouse challenge studies. Pneumolysin is released when the cells are
undergoing autolysis, this occurs late in infection, during the stationary phase of
growth. In addition to its hemolytic activity, pneumolysin activates the classical
pathway of complement. This is opposite of the effect the C3 degrading enzyme
and the Factor H binding protein have on the complement system. The hypothesis
for why both complement inhibiting and complement activating proteins would be
produced is that suppression of the complement system early in infection allows the
bacteria to replicate and then activation of the complement system later in the
infection induces inflammation and helps the bacteria to disseminate throughout the
host. ^\textsuperscript{2,44}

Autolysis of \textit{S. pneumoniae} is seen in older cultures and occurs late in
infection, during the stationary phase of growth. The protein responsible for
autolysis is autolysin. The autolysin is a surface protein that is activated during the
stationary phase of bacterial growth, it may also be important in cell division. This
enzyme breaks the bond between the NAM and alanine of the peptidoglycan. The
autolysin can only act if teichoic acid containing choline is present. \textsuperscript{21} It is thought
that the autolysin uses the choline to dock and then it can act on the peptidoglycan
bond. The autolysin produced by \textit{S. pneumoniae} is considered a virulence factor
because autolysin mutants are less virulent in mouse challenge studies. However,
antibody to autolysin is not protective in challenge studies in mice. An explanation
given for this is that autolysin contributes to virulence by causing the release of
intracellular virulence factors, e.g. pneumolysin, and autolysin antibody cannot neutralize this activity.\textsuperscript{2,44}

Another surface protein of \textit{S. pneumoniae} is called pneumococcal surface protein A (PspA). The proposed function for PspA is to decrease bacterial clearance from the blood. Mutants deficient in PspA are less virulent than wild type, and antibody against PspA is protective in challenge studies in mice.\textsuperscript{2,44}

\textbf{\textit{S. suis}}

Currently, there are no \textit{S. suis} proteins with a proven function in virulence. However, many \textit{S. suis} proteins that have been proposed to be involved in virulence. The two proteins that, for many years, were thought to be markers of virulence are extracellular factor (EF) and muramidase released protein (MRP). These two proteins were first identified by comparing protein profiles of \textit{S. suis} isolates from tissues of diseased pigs with protein profiles of isolates from tonsils of healthy pigs. EF and MRP were found in the \textit{S. suis} isolates from diseased pigs and not in most of the isolates from healthy pigs. The functions of EF and MRP are unknown, and the sequences do not have close homology to any known proteins. An MRP negative-EF negative mutant was not reduced in virulence compared to the parent. Therefore, it was concluded that EF and MRP were not required for virulence.\textsuperscript{62} The author proposed that these two proteins may contribute to virulence, but when absent, other factors also contribute to virulence so no loss in virulence is observed.

Another protein that has received recent attention as a potential virulence factor is the hemolysin. The hemolysin, suilysin, was described in 1994. It is a thiol-activated, oxygen labile, pore-forming extracellular toxin. It has similarities to
streptolysin O, and anti-streptolysin antibody inhibits the hemolytic activity of suilysin.\textsuperscript{16,24,28} Immunization of mice and pigs with suilysin induced protection against a homologous challenge.\textsuperscript{28} However, anti-suilysin antibodies were not detected in pigs recovered from experimental challenge with a hemolytic isolate.\textsuperscript{24} It is unclear whether suilysin has an antiphagocytic role and aids in survival of bacteria within a phagocyte, similar to other thiol-activated hemolysins expressed by other types of bacteria (e.g. listeriolyisin).

Some \textit{S. suis} isolates hemagglutinate red blood cells of a few species, horse and human.\textsuperscript{23,34} The hemagglutinating activity was found to be sensitive to protease treatment and to heat. Based on those results, it was proposed that the hemagglutinin was a protein; however, no protein with hemagglutinating activity has been identified. There was no correlation between hemagglutinating activity and virulence in mice. It is unknown what role the hemagglutinin plays in virulence, but it is thought it may contribute to adherence. Fimbriae are also present on all capsular types of \textit{S. suis}.\textsuperscript{23} Similar to hemagglutinin, the importance of the fimbriae has not been defined.\textsuperscript{23}

An adhesin for \textit{S. suis} was found on all isolates examined. The adhesin binds to galactosyl-(alpha 1-4)-galactose. The adhesin was immunogenic and induced in vitro bactericidal activity in blood. There was no correlation between the adhesin activity and the hemagglutinin activity. The authors propose that this adhesin should be investigated as a vaccine candidate since it was present on all isolates examined and it had good immunogenicity.\textsuperscript{67}
S. suis, like many other streptococci, has an IgG binding protein on its surface. It has been proposed that the IgG binding protein is a 52 kilodalton protein which is located on the cell surface and is also released extracellularly. The role in virulence is probably similar to that of other Fc receptors found on bacterial surfaces. They may camouflage the surface and interfere with opsonization and phagocytosis. However, there are no reports demonstrating the importance of this protein in virulence.

In mice challenge studies, it was observed that S. suis grown in broth with serum was more virulent than S. suis grown in the broth without serum. This led to the identification of an albumin binding protein on the surface of S. suis. The albumin binding protein is thought to be a 39 kilodalton protein. The authors proposed that the binding of albumin to the surface of S. suis may increase its uptake and survival of the S. suis in phagocytes. However, albumin bound on the surface of S. suis may act as a camouflage for the bacteria. The definitive role of this protein in virulence has not been established.

Considerable research is still required to define the role of S. suis proteins in virulence.

Summary

Streptococci have a wide variety of virulence factors. Research has provided important information for understanding the pathogenesis of streptococci, their means of evading the immune system, and what is required for a protective immune
response. This has led to a decrease in some of the diseases caused by streptococci. However, for many of the other streptococcal diseases a better understanding of the virulence factors of the bacteria is required before improved approaches to prevention and treatment can be designed.

The purpose of the research described in this dissertation was to gain a better understanding of \textit{S. suis}-induced disease and the interaction of \textit{S. suis} with pig PMNs.

\textbf{References}


CHAPTER 3. DISEASE INDUCTION AFTER INTRANASAL CHALLENGE WITH
STREPTOCOCCUS SUIS AND EVALUATION OF EFFICACY OF
STREPTOCOCCUS PORCINUS VACCINE AGAINST S. SUIS CHALLENGE

A paper to be submitted to Veterinary Microbiology

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Abstract

The natural route of exposure of swine to Streptococcus suis is oral/nasal; however, a reproducible model for inducing clinical disease by intranasal (IN) inoculation of healthy pigs with S. suis alone has not been published. The published models for experimental S. suis-induced disease are either intravenous or IN inoculation in pigs coinfected with another pathogen.\textsuperscript{9,15,21,25} In order to evaluate S. suis vaccine efficacy or the in vivo influence of S. suis virulence factors, an IN challenge model not involving coinfection is preferred. The overall objectives of the experiments reported here were 1) to develop an experimental S. suis challenge model that would not require a second pathogen and would mimic the route of exposure, clinical signs and lesions of the natural disease, 2) to use the IN challenge model to evaluate the efficacy of a Streptococcus porcinus vaccine in protection against S. suis-induced disease, and 3) to determine if in vitro passage reduced the virulence of the S. suis challenge strain.
The first experiment was designed to evaluate whether exposure to aerosolized *S. suis* would be better at inducing disease than IN inoculation, and whether irritation of the nasal mucosa by exposure to aerosolized ammonia prior to exposure to aerosolized *S. suis* would increase *S. suis*-induced disease. A *S. suis* isolate recovered from the meninges of a pig from a field case was used. The isolate was injected intravenously into a healthy pig. When this pig showed signs of central nervous system involvement, the brain and meninges were harvested, macerated, and frozen in aliquots for use as a stock culture to produce the challenge inocula. This stock culture was used for all the experiments in this work of study. The results of the first experiment indicated that direct intranasal administration of the *S. suis* after exposure to ammonia caused severe clinical disease which mimicked the natural *S. suis*-induced disease and produced a high mortality rate. Exposure to aerosolized *S. suis* produced less severe disease, and exposure to aerosolized ammonia, before exposure to aerosolized *S. suis*, did not increase the severity of the disease.

The second experiment was designed to evaluate the importance for disease induction of nasal mucosa irritation by exposure to aerosolized ammonia prior to IN inoculation of *S. suis*, and to evaluate the efficacy of a live avirulent *S. porcinus* vaccine against *S. suis* challenge. The results indicated that exposure to aerosolized ammonia before IN challenge with *S. suis* did not increase the severity of the disease induced. The pigs that received the live avirulent *S. porcinus* vaccine had a lower incidence of disease; however, the differences were not statistically significant.
It was concluded that *S. porcinus* vaccine was not a good candidate for inducing protection against *S. suis*-induced disease.

The third experiment was designed to determine if in vitro passage of the *S. suis* challenge strain would reduce its virulence. One group of pigs was exposed by IN inoculation to passage 1 *S. suis*. The other group was exposed by IN inoculation to the same isolate that had been serially passed on blood agar plates 20 times. There were no significant differences in incidence or severity of disease between these two challenge groups. Therefore, 20 passages in vitro did not effect the virulence of the *S. suis* serotype 2 isolate used as a challenge inoculum.

**General Introduction**

*Streptococcus suis* (*S. suis*) can colonize the tonsils of pigs at birth and is commonly isolated from clinically healthy pigs. In many pigs it remains in the tonsil without causing disease. However, in some pigs the bacteria invade causing a septicemia resulting in meningitis, arthritis, pericarditis, peritonitis, pleuritis, and/or pneumonia. *S. suis* can cause mortality rates of 5% to 15% in nursery age pigs. It is not understood why some isolates remain in a commensal relationship with the host, while others invade and kill the host. Often, *S. suis*-induced disease occurs when other stressors are present. For example, there is an increased incidence of *S. suis*-induced disease at weaning, when slurry gasses are high, and/or in pigs with porcine reproductive and respiratory syndrome virus (PRRSV).
The virulence factors for *S. suis* are poorly understood. Several potential virulence factors have been identified including capsule, muramidase-released protein, extracellular factor, hemolysin, and hemagglutinins. However, only capsule has been demonstrated by isogenic mutants to be required for virulence. Elimination of *S. suis* infection and prevention of *S. suis*-induced disease through management practices, medication, or vaccination have not been very successful.

In order to better understand the pathogenesis of *S. suis*-induced disease, and to evaluate virulence factors and new prevention and treatment regimes, challenge models of infection that, as closely as possible, mimic the natural route of exposure and disease process are required. The natural route of exposure to *S. suis* is predominately oral/nasal; therefore, invasion of the mucosal surface is an important step in the pathogenesis of the disease. A reproducible challenge model for inducing clinical disease with *S. suis* alone by experimental intranasal inoculation in a healthy pig has not been published. The published models for experimental production of *S. suis*-induced disease are intravenous or intranasal exposure to *S. suis* in pigs coinfected with pseudorabies virus, PRRSV, or *Bordetella bronchiseptica*. The intravenous model is useful for studying *S. suis* septicemia; however, it bypasses the process of *S. suis* colonization and invasion across the mucosal surface. The coinfection models are important for evaluation of pathogen interactions. These are valuable models for evaluating prevention and treatment regimes for coinfections. However, in order to evaluate *S. suis* vaccine
efficacy, or the in vivo influence of S. suis virulence factors, an intranasal challenge model not involving coinfection is preferred.

The objective of the research reported here was to develop an intranasal S. suis serotype 2 challenge model. A satisfactory model was defined as one that could cause clinical disease in at least 80% of the animals and not require coinfection with a second pathogen. The objectives of the individual experiments were: experiment 1) to determine if S. suis serotype 2, isolate ISU VDL #40634/94 induces clinical disease when administered by aerosolization and to determine whether irritation of the nasal mucosa using ammonia would increase the susceptibility of pigs to S. suis administered by aerosolization; experiment 2) to use an intranasal S. suis challenge model to evaluate the efficacy of Streptococcus porcinus as a vaccine to prevent S. suis-induced disease and to determine if aerosolized ammonia was needed in the intranasal challenge model; and experiment 3) to evaluate and compare the clinical disease caused by a virulent S. suis isolate before and after 20 passages in vitro.

Experiment 1

Introduction

S. suis serotype 2 was selected for use in the development of this challenge model because it is one of the serotypes commonly isolated from diseased animals. Based on reports that S. suis inoculated intranasally into healthy pigs would not
induce disease consistently, the culture was allowed to grow to maximal numbers.\textsuperscript{15,21} Pigs were exposed to \textit{S. suis} either by direct intranasal administration or by aerosolization in a closed chamber. The hypothesis was that aerosolized bacteria would mimic natural exposure and allow distribution of the bacteria throughout the respiratory tract. It was also hypothesized that exposure of pigs to ammonia fumes would mimic exposure to slurry gases and, therefore, increase the susceptibility of the pigs to \textit{S. suis}-induced disease without introduction of a second pathogen.

\textbf{Materials and Methods}

\textit{Animals}

Eighteen, clinically healthy four-week-old commercial crossbred pigs from a herd negative for PRRSV and with no known history of \textit{S. suis}-induced disease were used. Pigs were removed from the sows (weaned 12 to 18 days of age) and allowed to acclimate for six days. Each group was housed in a separate pen in the same airspace. Pigs were fed ad lib a nonmedicated commercial ration that met the National Research Council (NRC) requirements for the age and weight of the pigs. Tonsil swabs were obtained from each pig prior to \textit{S. suis} exposure and were screened for \textit{S. suis} serotype 2 (described below). Although \textit{S. suis} was recovered from the tonsils of almost all the pigs, none of the isolates tested were serotype 2.

\textit{Experimental design}

Three groups of six animals were used. One group was exposed in a chamber to aerosolized \textit{S. suis} only. The second group was exposed in a chamber
to aerosolized ammonia prior to exposure to aerosolized $S. \textit{suis}$. The third group was exposed in a chamber to aerosolized ammonia and then to $S. \textit{suis}$ by intranasal inoculation (Table 1). Rectal temperatures and clinical scores for central nervous system (CNS) signs, swollen joints/lameness, and lethargy were recorded daily. Moribund animals and animals still alive on the scheduled day for necropsy (Day 14 following challenge) were euthanized with Beuthanasia D™ (Schering-Plough) intravenously. All pigs were necropsied and tissues swabbed for bacterial isolation (described below).

\textit{S. suis challenge inoculum}

$S. \textit{suis}$ serotype 2 isolate ISU VDL#40634/94 was obtained from a field case of meningitis in a pig submitted to the Iowa State University (ISU) Veterinary Diagnostic Laboratory (VDL) in 1994 (case number 40634). The field isolate was grown in Todd-Hewitt broth (THB) (Difco) containing 5% heat inactivated fetal calf serum (hiFCS) for five hours at 37°C in 5% CO$_2$ air. The bacteria were pelleted by centrifugation and resuspended to a concentration of $10^{8.3}$ colony forming units (cfu)/milliliter (ml) in RPMI 1640. One ml was inoculated intravenously (IV) into a healthy weaned pig. When the pig began to show CNS signs (at 24 hours after IV inoculation), it was humanely euthanized. The infected brain and meninges were collected aseptically. A 10% homogenate of the brain and meninges was prepared in THB with 20% glycerol using a Stomacher 80 lab blender (Seward, Inc., England). One and one half ml aliquots were made and stored frozen at -80°C. This
brain/meninges homogenate was the stock material for preparing the experimental bacterial challenge inoculum used in all three experiments.

The brain/meninges homogenate was thawed and streaked, using a sterile cotton-tipped applicator, onto three plates of tryptose agar containing 5% bovine blood (BAP), and incubated for 15 hours at 37°C in 5% CO₂ air. Each plate contained approximately 300 colonies of S. suis. All the colonies on the BAPs were removed with a cotton swab and transferred to six ml THB containing 5% hiFCS. After a two hour incubation at 37°C in 5% CO₂, the six ml culture was added to 24 ml of warmed THB containing 5% hiFCS. After another two hour incubation, a 1:17 dilution of this culture was made in warmed THB containing 5% hiFCS. This culture was incubated an additional 3.5 hours then placed on ice until the pigs were inoculated (within 1 to 2 hours). The inocula were checked for purity by streaking onto a BAP and incubating at 37°C in 5% CO₂. The cfu/ml of bacteria in the culture was determined. The challenge inocula for all three experiments were prepared in this manner, unless otherwise described. Pigs receiving aerosolized S. suis were placed in a four by six by two feet chamber. The challenge inoculum was 30 ml of S. suis (1.6 x 10⁹ cfu/ml) aerosolized into the chamber for a 30 minute period. Pigs that received S. suis via direct intranasal inoculation were inoculated with 2 ml (1 ml/nostril) of 1.6 x 10⁹ cfu/ml.

Ammonia pretreatment

Ammonium hydroxide (1:5 dilution of reagent grade ammonium hydroxide, Fisher, Inc.) was aerosolized at 25 PSI using a nebulizer into a four by six by two
feet chamber containing six pigs. The ammonium concentration was approximately 100-150 ppm over a 30 minute period as determined by Sensidyne Gastec Detector Tube, (Animal Environment Specialists, Inc., Columbus, OH). The ammonia level in the chamber was then lowered by ventilation.

Observations and sampling

Rectal temperatures and clinical scores were recorded daily. Clinical scores were recorded for CNS signs (tremors, head tilt, convulsions, and paddling), swollen joints/lameness, and lethargy using a scale of 0-3 with 0 = normal, 1 = mild, 2 = moderate, and 3 = severe. Moribund animals and animals still alive on the scheduled day for necropsy were euthanized with Beuthanasia D™ (Schering-Plough) intravenously. The observations and sampling methods described here were used in all three experiments.

Necropsy

The tonsil, lung, pleura, peritoneum, pericardium, liver, spleen, joint(s), and brain/meninges were examined for macroscopic lesions. Swabs for bacterial culture were also collected from these tissues.

Bacteriology

Swabs from tissues obtained at necropsy and tonsil swabs obtained, prior to the study were swabbed onto BAPs. They were then incubated at 37°C in 5% CO₂ air for 24 to 48 hours. One colony from the cultures of internal tissues and three different colonies from the tonsil cultures were selected. Subcultures were tested for catalase reaction, Gram stain, Voges-Proskauer reaction, growth in 6.5% NaCl, and
production of amylase. The subcultures that were catalase negative, Gram stain positive, Voges-Proskauer reaction negative, did not grow in 6.5% NaCl, and were positive for the production of amylase were considered to be *S. suis*. All *S. suis* isolates were then checked for serotype 2 reactivity using the coagglutination test.\(^7,10\) This bacterial testing was done for experiments one and two. For experiment three, moderate to heavy growth of streptococcus-like, \(\alpha\)-hemolytic colonies were selected and subcultured. The subcultures were checked for serotype 2 reactivity using the coagglutination test.

**Statistical Analysis**

Differences in frequency of abnormal clinical signs, *S. suis* isolations, and mortality were analyzed by chi-square. This type of analysis was used for all three experiments.

**Results**

An animal was considered to have a fever if it had any recorded rectal temperature of \(\geq 104.5^\circ\text{F}\). The number of animals in each group that had a fever after inoculation with *S. suis* is recorded in Table 2. An animal was considered to have CNS infection if it had clinical signs of tremors, head tilt, convulsions and/or paddling, and/or *S. suis* isolation from the brain/meninges. An animal was considered to have joint infection if it had swollen joint(s), and/or lameness that did not exist before challenge, and/or *S. suis* isolation from the joint. The number of animals in each group with clinical signs or positive cultures from the CNS or joint(s), are also shown in Table 2. An animal was included in the last column of Table 2
(any evidence of infection) if it had any one of the following: fever, CNS signs, joint infection, lameness, lethargy, or *S. suis* cultured from any tissue other than tonsil.

Table 3 shows the number of animals in each group that had *S. suis* serotype 2 cultured from the various tissues swabbed at necropsy. A summary column of the total number of animals in each group that had *S. suis* serotype 2 cultured from an internal tissue (not tonsil) is also included in Table 3. Figure 1 presents the cumulative mortality in each group.

**Discussion**

All groups had at least 80% of the pigs with some evidence of infection. However, only one-half of the pigs in the aerosolized *S. suis* groups developed evidence of *S. suis* septicemia (isolation of *S. suis* from an internal organ). Irritation of the nasal mucosa using aerosolized ammonia did not enhance the disease caused by *S. suis* when the organism was administered by aerosolization into a chamber. *S. suis* administered intranasally following irritation of the nasal mucosa with ammonia gas caused severe *S. suis*-induced clinical disease. More animals had clinical disease, and *S. suis* was isolated from more tissues in animals inoculated intranasally compared to those exposed to aerosolized *S. suis*. An intranasal *S. suis* only group was not included in this experiment. Therefore, it was not determined if the aerosolized ammonia contributed to the disease progression in the pigs exposed to *S. suis* by intranasal inoculation.

The difference between the disease caused by the aerosolization and intranasal inoculation of *S. suis* exposures was probably due to a difference in dose
received by each animal. Aerosolization of the S. suis should help the S. suis pass deeper into the respiratory tract, but the dose received by each animal is unknown.

In comparison, intranasal inoculation introduces a large number of bacteria into the nasal and oral cavity. Based on the results of this study, it is important to have a large number of bacteria introduced into the nasal cavity. It does not appear to be important to have the S. suis aerosolized into small droplets for inhalation deep into the lungs. Therefore, in subsequent experiments for development of an intranasal S. suis only challenge model, S. suis was not aerosolized.

Experiment 2

Introduction

*Streptococcus porcinus* is a Group E Streptococcus that is capable of colonizing tonsils and causing cervical abscesses in pigs. In the 1970s an attenuated live S. porcinus vaccine was developed and found to be effective for prevention of cervical abscesses. Due to changes in swine management practices, the use of the attenuated live culture vaccine, and/or antibiotics in feed, S. porcinus-induced disease is now rarely observed. As a result, the vaccine is no longer used.

The protection that was provided by the attenuated live S. porcinus vaccine was thought to be due to an immune response to antigens present in many different serotypes, and not to serotype specific capsular antigens. S. suis is a group D streptococcus; however, it and may have virulence factors and important
immunogens similar to *S. porcinus*. The hypothesis tested in this experiment was that *S. porcinus* vaccine induces an immune response that protects pigs from developing *S. suis*-induced clinical disease.

There were two objectives for this study. One objective was to evaluate an attenuated live *S. porcinus* vaccine for efficacy against virulent *S. suis* administered intranasally. The other objective was to determine if ammonia pretreatment (as performed in experiment 1 above) was required for clinical disease to develop after intranasal inoculation of *S. suis*, isolate ISU VDL #40634/94.

**Materials and Methods**

*Animals*

Forty clinically healthy three- to four-week-old commercial crossbred pigs from a herd negative for PRRSV and with no known history of *S. suis*-induced disease were used. Pigs were removed from the sows (weaned 12 to 18 days of age) and allowed to acclimate to the isolation rooms for four days. Pigs were fed ad lib a nonmedicated commercial ration that met the NRC requirements for the age and weight of the pigs. All vaccinated pigs were in one room, and unvaccinated pigs were in another until the day of *S. suis* challenge. On the day of *S. suis* challenge, all pigs challenged with *S. suis* (both vaccinated and unvaccinated) were put in the same room, but in separate pens. All pigs not exposed to *S. suis* were housed in a separate room. Tonsil swabs were obtained from each pig prior to *S. suis* exposure, screened for *S. suis* serotype 2 (as described for experiment 1), and found to be negative.
Experimental design

Four groups of 10 pigs were used (Table 4). Group 1 received *S. porcinus* vaccination at three to four weeks of age. Two weeks following vaccination, group 1 was exposed in a chamber to aerosolized ammonia for 30 minutes (as described for experiment 1), and then inoculated intranasally with *S. suis*. Group 2 served as unvaccinated controls and was exposed in a chamber to aerosolized ammonia then inoculated intranasally with *S. suis*. Group 3 was part of the challenge model development. This group was challenged with *S. suis* intranasally, without exposure to aerosolized ammonia. Group 4 served as a negative control and did not receive any treatment. Rectal temperatures and clinical scores were recorded daily. Moribund animals and animals alive on the scheduled day for necropsy (14 days after *S. suis* exposure) were euthanized. The observations, samplings, bacteriology and necropsies were done as described in experiment 1.

*S. porcinus* vaccine

*S. porcinus* ATCC isolate #21223 was used to make the vaccine, and the vaccine was prepared by NOBL Laboratories, Inc., Sioux Center, Iowa. The *S. porcinus* avirulent live culture vaccine was grown in Brain Heart Infusion broth supplemented with 10% fetal calf serum. The culture was lyophilized and tested for sterility and purity. The bacteria were rehydrated to a dose of > 1 x 10^9 CFU per dose. Group 1 was vaccinated intranasally (1 ml/nostril) with 2 x 10^9 cfu of *S. porcinus* on Day 0 of the study. Pigs were three to four weeks of age when they were vaccinated.
S. suis challenge inoculum

S. suis serotype 2, isolate ISU VDL #40634/94 was prepared as described in Experiment 1. The titer of the S. suis inoculum was $1.4 \times 10^9$ cfu/ml. Pigs in Groups 1, 2, and 3 were exposed to S. suis serotype 2, isolate ISU VDL #40634/94 by intranasal inoculation of 1 ml of the bacteria in growth media into each nostril on Day 14 of the study.

Results

Effect of attenuated live S. porcinus vaccination

There were no abnormal clinical signs or elevated rectal temperatures recorded during the two-week observation period following S. porcinus vaccination that could be attributed to the vaccine. Joint swelling was observed in some vaccinated and nonvaccinated animals, suggesting it was probably not associated with vaccination. Ataxia was observed in one vaccinated animal beginning on Day 9 following vaccination. The animal was euthanized on Day 11 due to severe CNS signs; however, no gross lesions were found at necropsy, and there was no bacterial growth on cultures of meninges or other organs at necropsy. No histologic evidence of meningitis or encephalitis was found. No cause of clinical signs was identified.

Results following S. suis challenge

Vaccine efficacy. There were no significant differences between the vaccinated and unvaccinated pigs following challenge with intranasal S. suis (Tables 5 and 6). Both groups developed clinical signs of S. suis-induced disease and had S. suis cultured from similar internal tissues (Tables 5 and 6). The vaccinated group
had lower mortality (Figure 2) and a lower incidence of S. suis cultured from internal tissues than the unvaccinated group, however, the differences were not statistically significant.

**Challenge model development.** The unvaccinated pigs challenged by intranasal inoculation of S. suis, with or without preexposure to 100-150 ppm ammonia fumes in the air for 30 minutes, were compared based on clinical observations, rectal temperatures, mortality, and necropsy/bacteriology findings. Both groups showed mild/moderate lethargy in 90% of the pigs. Pigs that were exposed to aerosolized ammonia prior to challenge started to show clinical signs sooner than the group that were not exposed. The group that was exposed to aerosolized ammonia prior to challenge had peak clinical signs and fever on Days 2 and 3 following exposure to S. suis. The group that was not exposed to aerosolized ammonia had peak clinical signs and fever on Days 4 and 5 following challenge (data not shown). The mortality in the pigs that were exposed to aerosolized ammonia occurred on Days 3 and 5 following exposure to S. suis (Figure 2). Mortality in the pigs exposed to S. suis only occurred on Days 4, 6, and 9 after challenge. The onset of clinical signs and mortality both indicate a more rapid progression of disease in the pigs exposed to aerosolized ammonia; however, the overall outcome was not significantly different.

**Discussion**

Avirulent live culture S. porcinus intranasal vaccination did not provide adequate protection against S. suis serotype 2 clinical disease. Vaccination did
reduce the incidence of mortality, recovery of *S. suis*, and clinical signs after *S. suis* challenge. However, this was not statistically significant. Further development of this vaccine for protection against *S. suis*-induced disease was not pursued, since this level of protection was not acceptable.

Intranasal inoculation of *S. suis* with or without exposure to aerosolized ammonia produced similar clinical disease, fever, and mortality. The exposure to aerosolized ammonia resulted in a more rapid onset of disease. Based on the results of this and previous experiments, it was decided that the *S. suis* challenge model that was the least complex and still mimicked natural disease was intranasal administration of approximately $2 \times 10^9$ cfu of ISU VDL #40634/94 *S. suis* serotype 2 without any pretreatment required. With this model, clinical disease occurred in a majority of the animals. This model mimics the natural disease in that it requires the challenge bacteria to colonize and invade the mucosa. It should be a good model for evaluating *S. suis* pathogenesis, vaccine efficacy, and treatment regimes.

**Experiment 3**

**Introduction**

The previous experiments demonstrated that intranasally administered *S. suis* serotype 2 (isolate ISU VDL #40634/94) induced disease which mimicked naturally occurring disease. Previous publications reporting *S. suis* challenge models indicate that *S. suis* administered intranasally to healthy pigs does not consistently induce
disease similar to that observed in the field. The reason for the difference in these two observations is not clear. The challenge bacteria used in our previous experiments were preserved as a frozen homogenate of brain and meninges from a pig showing clinical signs of meningitis after intravenous challenge. It was hypothesized that the isolate we used was virulent because it was stored frozen as a brain/meninges homogenate and the challenge inoculum was produced without further passage. It was further hypothesized that in vitro passage of the bacteria would decrease its virulence in pigs. The objective of this study was to evaluate and compare the clinical disease caused by a virulent S. suis serotype 2 isolate ISU VDL #40634/94 before and after 20 passages in vitro on a solid media.

Materials and Methods

Animals and experimental design

Twenty commercial crossbred pigs from herds negative for PRRSV were used. Pigs were removed directly from the sows (weaned 12 to 18 days of age) and allowed to acclimate to the isolation rooms for 10 days. The pigs were injected intramuscularly with Excenel™ (Pharmacia & Upjohn Co., Kalamazoo, MI) once a day for three days immediately following their arrival at the isolation facilities. The pigs were inoculated intranasally with S. suis when they were between 22 and 28 days of age.

One group of 10 pigs received Passage 1 (P1) of S. suis serotype 2 isolate ISU VDL#40634/94 intranasally, and one group of 10 pigs received Passage 20 (P20) of S. suis serotype 2 isolate ISU VDL#40634/94 intranasally. The two groups
were housed on raised decks in separate isolation rooms. Rectal temperatures and clinical scores were recorded daily for each animal. Moribund animals and animals still alive on the day scheduled for necropsy were euthanized. At necropsy, tissues were swabbed and cultured for S. suis serotype 2. Observations, sampling, bacteriology and necropsies were performed as described in experiment 1.

_Bacteria_

_S. suis_ serotype 2, isolate ISU VDL #40634/94 was used as the challenge bacterium. This isolate was obtained and a stock culture prepared as described in experiment 1.

The P1 challenge inoculum was prepared as described in experiment 1. The P20 challenge inoculum was prepared by serially passaging the stock bacteria daily on BAPs for a total of 20 days. A single colony was picked each day and streaked onto a BAP. No morphologic changes were noted during the passaging. All colonies on day 20 were removed from the BAP with a cotton swab and placed in THB containing 20% glycerol, aliquoted to 1.2 ml volume, and then stored frozen at −80°C. P20 culture for inoculation of pigs was grown the same as described for P1. Pigs were inoculated intranasally with 2 ml (1 ml/nostril) of 1 x 10^9 cfu of bacteria per ml of growth media.

_Results_

The clinical disease caused by intranasal inoculation of P1 and P20 was similar (Table 7). Distribution of _S. suis_ in the tissues also was similar between the two groups (Table 8). There was 60% mortality prior to the scheduled day of
necropsy in the P20 group and 40% mortality in the P1 group (Figure 3). No significant (p>0.05) differences were detected between the two different challenge groups for any of the parameters measured.

Discussion

In vitro passaging is a common method for reducing the virulence of pathogens. However, 20 passages in vitro for S. suis isolate ISU VDL #40634/94 did not result in any detectable loss in virulence. It is unknown whether virulence would be altered with a higher number of passages, alternate media or different growth conditions. The working hypothesis was that the preservation of the stock challenge bacteria in frozen brain/meninges homogenate without passage contributed to its ability to induce disease when administered intranasally to healthy pigs. Based on the results of this experiment, the in vitro passaging of this isolate did not cause any loss in virulence. Therefore, it remains unclear as to why this challenge preparation of S. suis is apparently more successful at inducing disease after intranasal administration than other reported isolates.

Another important outcome of this experiment was that the P1 challenge results were similar to previous challenge results. This confirms our previous results with this challenge model and indicates that this model is reproducible.

The reason for the success of this challenge model is not clear. Some possibilities are: unique attributes of the isolate, the number of bacteria in the challenge inoculum, that the isolate was administered in growth media without washing, or the way the inoculum was grown.
This isolate may have some special attributes/virulence mechanisms that isolates used in previously published intranasal challenge studies did not have. It has been demonstrated that different isolates of \textit{S. suis} serotype 2 differ in their ability to produce disease. However, the basis for the differences is unknown. The isolate used in this research was recovered from the brain of a pig showing signs of meningitis. However, the ability to produce disease in a field case does not necessarily mean it will produce disease in an experimental setting. The DH5 strain used by Galina, et al and the D282 strain used by Vecht, et al were both recovered from the brain of a pig showing signs of meningitis and under their challenge conditions neither strain caused clinical signs in healthy pigs.

The titer of the challenge inoculum may contribute to the success of this challenge model. A titer of $10^{9.3}$ cfu per pig was used. This titer is higher than the $10^7$ cfu per pig titer used by Galina, et al\cite{9} and higher than the $2 \times 10^6$ cfu per pig titer used by Vecht, et al.\cite{21} No clinical disease was observed following intranasal challenge with \textit{S. suis} at those titers. Iglesias, et al used a titer of $10^9$ cfu per pig.\cite{15} In that study, 50% of the pigs showed clinical signs of \textit{S. suis}-induced disease and bacteria were cultured from internal tissues of those animals; however, there were no CNS signs induced and no mortality. In a separate study by Thanawongnuwech, et al\cite{19}, using isolate ISU VDL #40634/94 grown using the same method described in experiment 1 of this paper, the $10^9$ cfu per ml culture was diluted 1:10 using Hank's Balanced Salt Solution. When the diluted culture of $10^8$ cfu per ml was inoculated intranasally into healthy pigs, only 14% developed clinical \textit{S. suis}-induced disease.
This indicated that either the titer of bacteria or inoculation in undiluted growth media may be important. The titer of $10^8$ cfu per ml was only achieved when heat inactivated fetal calf serum was added to the Todd Hewitt broth, and the bacteria were grown using several feeding steps. This method of growth may contribute to the virulence of the bacteria. Iglesias et al.\textsuperscript{15}, reported that the challenge bacteria used was grown on agar and then in broth, but the times were not included. The growth conditions for the bacteria used by Galina et al.\textsuperscript{9}, were not described. Vecht et al.\textsuperscript{21}, grew the bacteria on agar then in broth for 18 hours and then added fresh broth and incubated for an additional 6 hours. Since, all of the methods for growing the bacteria were different it is possible that this influenced the outcome of intranasal S. suis challenge.

Another possibility for the virulence of the challenge model reported here is that there are important virulence factors in the growth media that allow the bacteria to successfully colonize and invade. In the published reports of S. suis intranasal challenge, it is not clear if the bacteria were administered in growth media or if the bacteria were washed before administration.\textsuperscript{9,15,21} As mentioned above, when ISU VDL #40634/94 was grown to $10^8$ cfu and then diluted 1:10 in Hank's Balanced Salt Solution, the in vivo virulence was greatly reduced.\textsuperscript{19}

Finally, the susceptibility of the pig may contribute to the differences observed. We challenged five to six week old pigs that were weaned directly from the sow into isolation facilities. In the other reports, pigs were 9 weeks old, 7 days old, or 3 weeks old. The 7 day old pigs were cesarean derived pigs and did not
receive colostrum\textsuperscript{21} so maternal antibody interference cannot explain their lack of susceptibility. The pigs challenged at 3 weeks of age may have had maternal antibody that interfered with their susceptibility to \textit{S. suis}-induced disease.\textsuperscript{9} Some of the 9-week-old pigs were susceptible to the \textit{S. suis} alone (50% clinical disease)\textsuperscript{15}; the increased age may have decreased the susceptibility of some of the pigs in that experiment compared to younger pigs used in these studies.

Further study is required to determine why this experimental intranasal \textit{S. suis} challenge model is successful in inducing disease. Evaluation of challenge with $10^6$ cfu of \textit{S. suis} in PBS compared to $10^9$ cfu in growth media would indicate the importance of factors in the growth media. A study comparing different concentrations of \textit{S. suis} in growth media as the challenge inoculum would help clarify the importance of the challenge titer. It would be interesting to obtain other isolates and grow them like the ISU VDL \#40634 isolate is grown to see if the method of growing the bacteria influences the in vivo virulence of the isolates. Finally, challenge of different ages of pigs obtained from the same source would provide information regarding age influence on susceptibility.

**Summary and Conclusions**

A reproducible intranasal \textit{S. suis} challenge model is described. Clinical signs of \textit{S. suis}-induced disease begin a few days after intranasal inoculation, indicating that colonization and invasion are part of this model. The resulting disease mimics
naturally occurring \textit{S. suis} induced clinical signs and lesions. This model is successful without aerosolization of the bacteria. Irritation of the nasal mucosa using aerosolized ammonia is not needed nor is coinfection with a predisposing pathogen. Twenty passages in vitro does not change the virulence of this isolate. Based on comparisons to other reports, it appears that titer and/or growth media in the inocula is important for intranasal challenge to induce disease.$^{9,15,19,21}$

Finally, using this intranasal \textit{S. suis} challenge model, \textit{S. porcinus} avirulent live vaccine was evaluated for efficacy against \textit{S. suis}-induced disease. The \textit{S. porcinus} vaccine reduced \textit{S. suis}-induced disease incidence, but not significantly. This degree of efficacy was judged to be inadequate to pursue it as a vaccine for protection against \textit{S. suis}-induced disease.

\textbf{Acknowledgements}

The authors wish to thank Dr. Dagmar Frank and Tom Skadow for their technical assistance, and the Iowa State University Diagnostic Bacteriology Laboratory for their assistance and consultations. This work was supported in part by the Iowa Livestock Health Advisory Council and NOBL Laboratories, Inc.
References


Table 1. Experimental design for experiment 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of pigs</th>
<th>Pretreatment</th>
<th>Route of <em>S. suis</em> administration</th>
<th>Necropsy (days after challenge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>None</td>
<td>Aerosolized in a chamber</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>Ammonia fumes</td>
<td>Aerosolized in a chamber</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>Ammonia fumes</td>
<td>Intranasal inoculation</td>
<td>14</td>
</tr>
</tbody>
</table>
Table 2. Number of pigs in each group from experiment 1 that had evidence of fever, CNS infection, joint infection, or any of these three signs and/or lethargy after *S. suis* challenge exposure.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fever (≥ 104.5°F)</th>
<th>CNS Infection (clinical signs and/or culture positive)</th>
<th>Joint Infection (clinical signs and/or culture positive)</th>
<th>Number of Animals with Evidence of <em>S. suis</em> infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerosolized <em>S. suis</em></td>
<td>4/6</td>
<td>3/6</td>
<td>1/6</td>
<td>5/6</td>
</tr>
<tr>
<td>NH₃ pretreatment + aerosolized <em>S. suis</em></td>
<td>4/6</td>
<td>2/6</td>
<td>1/6</td>
<td>5/6</td>
</tr>
<tr>
<td>NH₃ pretreatment + intranasal <em>S. suis</em></td>
<td>5/6</td>
<td>5/6</td>
<td>4/6</td>
<td>5/6</td>
</tr>
</tbody>
</table>

There were no significant differences between groups.
Table 3. Number of pigs in each group from experiment 1 that were culture positive for *S. suis* serotype 2 from tissues swabbed at necropsy.

<table>
<thead>
<tr>
<th>Group</th>
<th>Tonsil</th>
<th>Lung</th>
<th>Plra</th>
<th>Perit</th>
<th>Peric</th>
<th>Liver</th>
<th>Spleen</th>
<th>Joint</th>
<th>CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerosolized <em>S. suis</em></td>
<td>3/6</td>
<td>3/6</td>
<td>1/6</td>
<td>1/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>1/6</td>
<td>2/5</td>
</tr>
<tr>
<td>NH₃ pretreatment + aerosolized <em>S. suis</em></td>
<td>3/6</td>
<td>3/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
<td>1/6</td>
<td>2/6</td>
</tr>
<tr>
<td>NH₃ pretreatment + intranasal <em>S. suis</em></td>
<td>5/6</td>
<td>5/6</td>
<td>1/3</td>
<td>2/6</td>
<td>2/6</td>
<td>4/6</td>
<td>2/6</td>
<td>4/6</td>
<td>5/6</td>
</tr>
</tbody>
</table>

Pra = pleura  
Perit = peritoneum  
Peric = pericardium  
There were no significant differences between groups.
**Table 4. Experimental design for experiment 2.**

<table>
<thead>
<tr>
<th>Group</th>
<th># of pigs</th>
<th>Vaccination</th>
<th>Pretreatment</th>
<th>Intranasal Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>S. <em>porcinus</em></td>
<td>Aerosolized Ammonia</td>
<td>S. <em>suis</em></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>None</td>
<td>Aerosolized Ammonia</td>
<td>S. <em>suis</em></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>None</td>
<td>None</td>
<td>S. <em>suis</em></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>
Table 5. Number of pigs in each group from experiment 2 that had evidence of fever, CNS infection, joint infection, or any of these three signs and/or lethargy after *S. suis* challenge.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fever (≥ 104.5 °F)</th>
<th>CNS Infection (clinical signs and/or culture positive)</th>
<th>Joint Infection (clinical signs and/or culture positive)</th>
<th>Number Animals with Evidence of <em>S. suis</em> Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated, NH₃ pretreatment + <em>S. suis</em> IN</td>
<td>4/9ᵃ</td>
<td>2/9</td>
<td>1/9</td>
<td>9/9ᵇ</td>
</tr>
<tr>
<td>Unvaccinated, NH₃ pretreatment + <em>S. suis</em> IN</td>
<td>6/10ᵇ</td>
<td>4/10ᵇ</td>
<td>1/10</td>
<td>9/10ᵇ</td>
</tr>
<tr>
<td>Unvaccinated, <em>S. suis</em> IN</td>
<td>7/10ᵇ</td>
<td>5/10ᵇ</td>
<td>2/10</td>
<td>9/10ᵇ</td>
</tr>
<tr>
<td>Control</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

ᵃ p ≤ 0.05 compared to control  
ᵇ p ≤ 0.01 compared to control  
There were no significant differences between vaccinated and unvaccinated animals that were challenged with *S. suis*. 
Table 6. Number of pigs in each group from experiment 2 that were culture positive for *S. suis* serotype 2 from tissues swabbed at necropsy.

<table>
<thead>
<tr>
<th>Group</th>
<th>S. suis from internal tissue</th>
<th>Tonsil</th>
<th>Lung</th>
<th>Pira</th>
<th>Perit</th>
<th>Peric</th>
<th>Liver</th>
<th>Spleen</th>
<th>Joint</th>
<th>CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated, NH₃ pretreatment + <em>S. suis</em> IN</td>
<td>2/9</td>
<td>6/9ᵇ</td>
<td>1/9</td>
<td>0/9</td>
<td>0/9</td>
<td>2/9</td>
<td>0/9</td>
<td>2/9</td>
<td>1/9</td>
<td>2/9</td>
</tr>
<tr>
<td>Unvaccinated, NH₃ pretreatment + <em>S. suis</em> IN</td>
<td>4/10ᵃ</td>
<td>5/10ᵃ</td>
<td>3/10</td>
<td>0/10</td>
<td>0/10</td>
<td>2/10</td>
<td>0/10</td>
<td>2/10</td>
<td>0/10</td>
<td>4/10ᵃ</td>
</tr>
<tr>
<td>Unvaccinated, <em>S. suis</em> IN</td>
<td>5/10ᵃ</td>
<td>1/10</td>
<td>2/10</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10</td>
<td>0/10</td>
<td>1/10</td>
<td>2/10</td>
<td>5/10ᵃ</td>
</tr>
<tr>
<td>Control</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

Pira = Pleura
Perit = Peritoneum
Peric = Pericardium
ᵃᵇ<p < 0.05 compared to control
ᵇᵇ<p < 0.01 compared to control

There were no significant differences between vaccinated and unvaccinated animals that were challenged with *S. suis*. 

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67
Table 7. Number of pigs in each group from experiment 3 that had evidence of fever, CNS infection, joint infection, or any of these three signs and/or lethargy after *S. suis* challenge exposure.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fever (≥ 104.5 °F)</th>
<th>CNS Infection (clinical signs and/or culture positive)</th>
<th>Joint Infection (clinical signs and/or culture positive)</th>
<th>Any evidence of <em>S. suis</em> Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. suis</em> P1</td>
<td>9/10</td>
<td>6/10</td>
<td>8/10</td>
<td>10/10</td>
</tr>
<tr>
<td><em>S. suis</em> P20</td>
<td>8/10</td>
<td>7/10</td>
<td>7/10</td>
<td>10/10</td>
</tr>
</tbody>
</table>

There were no significant differences between the groups.
Table 8. Number of pigs in each group from experiment 3 that were culture positive for *S. suis* serotype 2 from tissues swabbed at necropsy.

<table>
<thead>
<tr>
<th>Group</th>
<th>S. suis from Internal Tissue</th>
<th>Lung</th>
<th>Pleura</th>
<th>Peritoneum</th>
<th>Pericardium</th>
<th>Joint</th>
<th>CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. suis</em> P1</td>
<td>8/10</td>
<td>2/10</td>
<td>1/10</td>
<td>2/10</td>
<td>2/10</td>
<td>2/8</td>
<td>6/10</td>
</tr>
<tr>
<td><em>S. suis</em> P20</td>
<td>7/10</td>
<td>1/10</td>
<td>3/10</td>
<td>2/10</td>
<td>0/10</td>
<td>0/8</td>
<td>7/10</td>
</tr>
</tbody>
</table>

There were no significant differences between the groups.
Figure 1. Cumulative mortality of animals in each treatment group per day of experiment 1.
Figure 2. Cumulative mortality of animals in each treatment group per day of experiment 2 following S. suis inoculation on Day 14.
Figure 3. Cumulative mortality of animals in each treatment group per day of experiment 3.
CHAPTER 4. CHARACTERIZATION OF A NEUTROPHIL SUPPRESSIVE FACTOR FROM STREPTOCOCCUS SUIS

A paper to be submitted for publication

Gayle B. Brown and James A. Roth

Abstract

*Streptococcus suis* (*S. suis*) commonly colonizes the tonsils of healthy pigs. However, it is capable of invading and causing septicemia, meningitis, arthritis, serositis and/or pneumonia. Some potential virulence factors of *S. suis* have been identified but their roles in evasion of immune destruction and induction of disease are not understood. The objective of this study was to begin to identify factors that inhibit neutrophil function and that may be important in the pathogenesis of *S. suis*-induced disease. *S. suis*, serotype 2, isolated from the meninges of a pig showing signs of meningitis was inoculated into Todd-Hewitt broth and grown for 18 hours at 37°C in 5% CO₂ air. The bacteria were then pelleted, washed twice with PBS and resuspended in 2 ml of PBS. The resuspended bacteria were incubated for two hours at 37°C in 5% CO₂ air. The bacteria were pelleted and the supernatant collected and filtered through a 0.2 micron filter. Porcine neutrophil iodination, cytochrome C reduction, *Staphylococcus aureus* ingestion, and random migration under agarose assays were performed with supernatant added to the assays at 10% of the final volume and PBS as a control. *S. suis* supernatant suppressed the iodination reaction to 49% of control, the other three neutrophil function assays were
not altered, or were only mildly suppressed. The suppressive activity detected by the iodination assay was heat stable, was not present in a capsular preparation and was not present in supernatant prepared by incubating *S. suis* at 4°C instead of 37°C. When the supernatant was passed through a 1000 dalton cut-off membrane filter the filtrate suppressed the iodination reaction to 61% of the PBS control. The 1000 dalton filtrate was fractionated using reverse phase-high performance liquid chromatography and the suppressive activity was found in one of the fractions collected. The suppressive fraction contained guanine and at least two other unknown components that may be other bases and/or nucleosides. The exact molecular structure, mechanism of action and contribution to *S. suis* virulence of the neutrophil suppressive factor remains to be determined.

**Introduction**

*Streptococcus suis* (*S. suis*) is an alpha-hemolytic gram positive organism that can induce disease in pigs, humans, cattle, dogs and other animals. S. suis-induced disease is predominately a problem in young pigs, especially nursery age, but it can cause disease in any age pig. Meningitis, arthritis, septicemia and/or pneumonia characterize the disease.

*S. suis* colonizes the tonsils of healthy pigs. The proposed pathogenesis of the disease is that monocytes phagocytose virulent *S. suis* organisms from the tonsils. The organisms survive and replicate in the monocyte, and are distributed systemically by the monocytes. Once spread systemically they are released from
their intracellular location, evade ingestion and killing by the polymorphonuclear cells (PMNs) and induce clinical disease.\textsuperscript{23}

Potential virulence factors described for \textit{S. suis} include capsule, suilysin (a hemolysin), albumin-binding protein, immunoglobulin-binding protein, muramidase released protein, extracellular factor, hemagglutinins, and an adhesin.\textsuperscript{4,7,11-16,20-22} The capsule is the only factor that has been found to be critical for virulence.\textsuperscript{4} The role of the capsule in virulence is thought to be inhibition of phagocytosis and possibly increased survival in phagocytes.\textsuperscript{1} Capsular thickness shows some correlation to resistance to PMN killing.\textsuperscript{17} However, resistance to PMN killing was observed in an isolate that did not have a thick capsule suggesting that other factors are involved in resistance to PMN killing.\textsuperscript{17}

The interaction of \textit{S. suis} with PMNs is clearly important in the pathogenesis of the disease. PMNs are part of the early defense against \textit{S. suis} infection. Many virulence factors of bacteria are capable of inhibiting PMN function. The objective of this research was to begin to characterize and identify virulence factors of \textit{S. suis} that interfere with phagocyte killing. Instead of comparing differences between virulent and avirulent isolates of \textit{S. suis} in order to discover virulence factors, a virulent isolate was used and its effect on PMN function was monitored. A neutrophil suppressive molecule was identified and found to be a small molecule that decreases the myeloperoxidase (MPO)-H\textsubscript{2}O\textsubscript{2}-halide bactericidal mechanism of porcine neutrophils.
Materials and Methods

S. suis serotype 2 isolate

The bacteria used for this research was S. suis serotype 2 isolate ISU-VDL #40634/94 described previously.2 Briefly, the isolate was first cultured by the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) from the meninges of a field case of a pig showing signs of meningitis. The isolate was inoculated intravenously into another pig. When the pig showed signs of meningitis the brain/meninges were harvested and homogenized. The brain/meninges homogenate is stored frozen at -80°C.

S. suis supernatant

S. suis serotype 2 (1.4 x 10^3 cfu) in the brain/meninges homogenate was inoculated into 40 ml of Todd-Hewitt Broth (THB) (Difco) and incubated for 18 hours at 37°C in 5% CO₂ air. The bacteria were then pelleted, washed twice in phosphate buffered saline (PBS) and then resuspended in two ml of PBS. The resuspended bacteria were incubated for two hours at 37°C in 5% CO₂ air. The bacteria were then pelleted and the supernatant collected and filtered through a 0.2 micron filter.

Heat stability of the supernatant was determined by heating the supernatant to 121°C for 15 minutes. To determine if capsule was responsible for the suppression of the iodination reaction, a supernatant was prepared as described above with minor modifications. After washing with PBS, the pellet was resuspended in PBS, then boiled for 15 minutes to allow the release of capsular material into the supernatant. The cells were pelleted and the supernatant collected
and filtered as described above. To determine whether the *S. suis* needed to be at physiologic temperature in order to release the suppressive factor extracellularly, the supernatant was prepared as described above except that instead of a two hour incubation at 37°C the *S. suis* in PBS was incubated at 4°C.

**Fractionation using molecular weight filters**

The 0.2 micron filtered supernatant was passed through a 10,000 dalton cut-off membrane by centrifugation (Amicon CP10, Amicon, Beverly, MA). The filtrate that passed through the 10,000 dalton cut-off membrane was tested and found to contain the neutrophil suppressive activity. Using a stirred cell system, this filtrate was then passed through a 1000 dalton cut-off membrane (Millipore, Bedford, MA) and the filtrate collected (1000 dalton filtrate). This filtrate was checked for suppressive activity on porcine PMNs and the remainder was stored at -80°C.

**Reverse phase-high performance liquid chromatography**

The 1000 dalton filtrate was separated (100 µl aliquots) on a C18 column (Jupiter 300, 5 µm particle size, 4.6 X 250 mm, Phenomenex, Torrance, CA) using a Beckman 12114M pump and a 406 Analog Interface Module (Beckman Instruments, Inc., Palo Alto, CA). One half minute timed fractions over 30 minutes were collected with a gradient of acetonitrile (0-30%) in 0.1% trifluoroacetic acid with a flow rate of 0.5 ml/min. Fractions were collected (SC100 fraction collector, Beckman Instruments, Inc., Palo Alto, CA) and pooled with similar fractions of previous runs, evaporated to dryness under vacuum, dissolved in PBS and tested for activity, or analyzed for composition.
Amino acid analysis

Amino acid analysis was performed by the ISU Protein facility using a Perkin Elmer Applied Biosystems Model 420 Derivatizer/Analyzer Amino Acid Analysis System (Foster City, CA). Samples were hydrolyzed at 150°C with 6N HCL using a PicoTag workstation (Waters), derivatized with phenylisothiocyanate (PITC) and separated on a C18 silica column. The derivatized amino acids were detected at 254 nm.

Amino acid sequence determination

A 492 Procise Protein Sequencer (Perkin Elmer Applied Biosystems, Inc. Foster, CA) was used to attempt to determine an amino acid sequence. This sequencer uses the Edman degradation method of sequencing.

Molecular weight determinations

Determination of the molecular weight was attempted using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI) (Finnigan MAT Lasermat 2000, Hemel Hempsted, England). The sample was loaded onto a sample spot with matrix and ionized with pulsed nitrogen laser at 337 nm.

PMN isolation

Porcine neutrophils were isolated from whole blood that was collected into acid-citrate-dextrose anticoagulant. The anticoagulated blood was centrifuged for 20 minutes at 700 x g. The plasma was discarded and the red blood cells were lysed by flash lysis with cold phosphate buffered deionized water; isotonicity was restored by adding 2.7% sodium chloride. The remaining cells were resuspended in Hanks buffered saline solution without Ca++, Mg++ or phenol red (HBSS) (GibcoBRL,
Grand Island, NY) and layered over Histopaque 1.077 (Sigma, St. Louis, MO). After centrifugation at 525 x g for 30 minutes the polymorphonuclear cells (PMNs) were found in the pellet with the remaining red blood cells. The remaining red blood cells were lysed as described above. The PMNs were washed with HBSS and then resuspended in HBSS to a concentration of 5.0 x 10⁷ PMN per ml for use in the neutrophil function assays.

**Neutrophil function assays**

The assays used were iodination, cytochrome C reduction, *Staphylococcus aureus* ingestion and random migration under agarose. These assays were performed as previously described.⁶,¹⁰,¹⁸ The effect of the *S. suis* supernatant on these assays was evaluated by comparing to a PBS control and calculating the percent of PBS control.

The *iodination assay* evaluates the influence of *S. suis* virulence factors on the activity of the myeloperoxidase-hydrogen peroxide-halide (MPO-H₂O₂-halide) antibacterial system of the neutrophil. The standard reaction mixture for the determination of stimulated iodination contained 2.5 x 10⁶ PMNs, 0.05 µCi ^125^I, 20 nmole Na^1, 0.5 mg of opsonized zymosan, 50.0 µl of either *S. suis* supernatant or PBS and 325 µl of Earle's balanced salts solution. The reaction mixture was incubated 20 minutes at 37°C, and the reaction was stopped by addition of 3.0 ml of cold 10% trichloroacetic acid. The resulting precipitate was washed with cold trichloroacetic acid and the amount of radioactivity in the precipitate determined in a gamma counter. The incorporation of Na^1 was expressed as nmole of Na^1/10⁷ PMNs/hr. The assay was performed in duplicate.
The cytochrome C reduction assay was used to evaluate the influence of the potential S. suis virulence factor on the production of superoxide anion during the burst of oxidative metabolism by the neutrophil. The standard reaction mixture for the determination of the influence of S. suis supernatant on this function contained 2.5 x 10^6 PMNs, 0.5 mg of opsonized zymosan as a stimulant, and 30 µl of either S. suis supernatant or PBS in 300 µl of a 0.32 mM solution of cytochrome C in HBSS. The assay was performed in duplicate in microtiter plates. The reaction was allowed to proceed for 5 minutes at 37°C. The optical density (OD) was determined by the difference of the OD at 550 nm and 650 nm using a micro-ELISA plate reader.

The S. aureus ingestion assay was used to evaluate the influence of S. suis supernatant on the ability of porcine neutrophils to ingest bacteria. The standard reaction mixture contained 100 µl of 125I-labeled heat-killed S. aureus, 50 µl of neutrophils (2.5 x 10^7), (bacteria to PMN ratio = 60:1), 50 µl of a 1:4 dilution of anti-S. aureus serum, 300 µl of Earle's BSS, and 50 µl of either S. suis supernatant or PBS as a control. The reaction was incubated at 37°C for 10 minutes, after which the non-phagocytized S. aureus was removed by adding 0.5 units of lysostaphin (Sigma) to each tube. The PMNs were washed twice and the amount of PMN-associated radioactivity was determined. The percentage of 125I-labeled S. aureus ingested was calculated. The assay was performed in duplicate.

Migration under agarose was evaluated by adding 10 µl of S. suis, or PBS as a control, to 90 µl of the standard PMN suspension, then 10 µl of that mixture was added to wells cut in agarose (bicarbonate-buffered Medium 199 with Earle's salts
containing 0.8% antibiotic). The agarose plates were incubated 18 hours in a humidified incubator containing 5% CO₂ air. Following incubation, the cells were fixed with glutaraldehyde, stained with crystal violet, and the area of PMN migration (in mm²) away from the wells was measured. The assay was performed in duplicate.

Results

Characterization of *S. suis* supernatant

Unfractionated *S. suis* supernatant suppressed iodination activity of porcine PMNs to 48.7% of the PBS control value. The cytochrome C reduction, *S. aureus* ingestion and migration under agarose activities were not affected, or were only mildly affected (Table 1). Since the iodination assay was significantly suppressed, this assay was used to monitor for the presence of neutrophil suppressive activity. Suppression of the iodination reaction by *S. suis* metabolic supematant after heating at 121°C for 15 minutes was unchanged (50.0 % of PBS control ± 6.5, SEM). Preparing the supematant using a 4°C incubation temperature for 2 hours, instead of 37°C, resulted in supematant that did not suppress the iodination reaction (100.6% of PBS control ± 8.5, SEM). The supematant prepared by boiling the *S. suis* cells was only mildly suppressive to PMN iodination activity (86.7% of PBS control ± 2.7 SEM). Colony counts before and after the 2 hour 37°C incubation in PBS were not significantly different, indicating there was no cell death causing the release of suppressive factors.
Fractionation of *S. suis* metabolic supernatant

The 10,000 dalton filtrate had similar suppressive activity as the unfractionated supernatant and the 1000 dalton filtrate also had suppressive activity similar to the unfractionated metabolic supernatant (45.6% ± 3.1 and 61.3% ± 4.2, respectively).

The 1000 dalton filtrate was fractionated using RP-HPLC (Figure 1). The fractions containing major peaks were checked for their ability to suppress neutrophil iodination reaction (Table 2). Fraction 32 suppressed neutrophil iodination reaction similar to the unfractionated metabolic supernatant and was further evaluated for composition.

Determination of the composition of fraction 32

Amino acid analysis results were inconclusive and attempts to determine the amino acid sequence of fraction 32 were not successful. However, during the amino acid analysis, a large peak that did not correlate with any of the amino acid standards was identified in the C-18 RP-HPLC separation of the hydrolyzed and derivatized sample. In order to determine the composition of this peak, a hydrolyzed sample of fraction 32 sample was injected into a reversed-phase C18 column and the sample eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid (0-100%) over 50 minutes at 200 μl per minute. The chromatogram revealed three peaks and is shown in Figure 2. When a guanine standard was injected into and eluted from the C18 column in the same manner as hydrolyzed fraction 32, the guanine had a similar elution time as peak 2 (Figure 2). MALDI mass spectrometry was done on each of the three peaks shown in Figure 2. Peak 2 had a MALDI signal at m/z =
152; the guanine standard also produced a MALDI signal at m/z = 152. Peaks 1 and 3 have not been positively identified but MALDI results suggests that Peak 3 may be a form of guanine. At low laser energies it has a MALDI signal at m/z = 225 and at higher laser energies that may cause breakdown of the molecule, there is a signal at m/z = 152 where the guanine standard gives a signal. Peak 1 has a MALDI signal at m/z = 112. This would be where cytosine would be expected to produce a signal, so it is possible that peak 1 contains cytosine.

The MALDI of the unhydrolyzed fraction 32 was inconclusive. The molecular weight of the entire unhydrolyzed fraction remains unknown.

**Discussion**

Metabolically active *S. suis* releases a factor into the supernatant that passes through a 1000 dalton cut-off membrane filter and suppresses porcine neutrophil MPO-H$_2$O$_2$-halide antibacterial activity but not neutrophil oxidative burst, ingestion or migration under agarose. It is heat stable and is not present in a capsular preparation. The *S. suis* must be metabolically active to release the neutrophil suppressive factor into the supernatant. The suppressive factor appears to be composed of guanine and at least two other components that may be other bases and/or nucleosides.

Neutrophils are important in host protection against extracellular bacterial infections and are important in host defense against *S. suis*. Under certain circumstances, *S. suis* invades the host and evades immune destruction by
neutrophils resulting in disease. The objective of this study was to determine if S. suis secretes products extracellularly that inhibit important bactericidal activities of neutrophils. Important functions of neutrophils include migration to the site of infection, phagocytosis, oxidative burst and production of oxygen radicals, degranulation and release of enzymes and cationic peptides, and the MPO-H₂O₂-halide system. Myeloperoxidase is released by the granules and catalyzes the reaction of H₂O₂ and halides resulting in the production of hypohalides. These hypohalides oxidize bacterial proteins.

The assays used in this study indirectly measure the neutrophil migration, phagocytosis, oxidative burst, and the MPO-H₂O₂-halide system by measuring migration under agarose, S. aureus ingestion, cytochrome C reduction and iodination of proteins, respectively. The porcine neutrophil suppressive factor only suppressed the MPO-H₂O₂-halide system. Since the oxidative burst was not affected, it is likely that the production of H₂O₂ was not affected. Therefore, the suppressive factor may be affecting the degranulation of myeloperoxidase or its catalytic activity.

Virulent S. suis is able to survive in phagocytic cells. It is possible that the suppressive factor identified here contributes to the survival of the S. suis within the phagocytes by decreasing degranulation into phagosomes.

Purines and nucleosides are known to affect neutrophil function. Guanine, guanine monophosphate and adenine released by Brucella abortus and Haemophilus somnus are known to suppress bovine PMN iodination activity. These are facultative intracellular bacteria and it is proposed that the release of the
purines and nucleosides allow the bacteria to evade destruction in the neutrophil. The bovine neutrophil suppressive factor was found in the metabolic supernatant of *H. somnus* similar to *S. suis*. For *B. abortus* the purines and nucleosides were not released into the supernatant until the bacteria were heated.

Receptors for adenosine are found on human neutrophils. The binding of adenosine to the A2 adenosine receptor of human neutrophils results in decreased oxidative burst, degranulation and decreased binding of activated neutrophils to endothelial cells because of decreased up-regulation of beta 2 integrins and decreased loss of L-selectins on the neutrophil surface. Guanosine is a purine like adenosine; however, it does not seem to affect human neutrophils in the same manner as adenosine. It is not known if human neutrophils have receptors for guanosine. It is possible that bovine and pig neutrophils have receptors for guanosine and that binding of this receptor results in the decreased myeloperoxidase-H$_2$O$_2$-halide reaction. It is not known whether porcine and bovine neutrophils have adenosine receptors. Possibly these species have a receptor that will bind either of the purine nucleosides. It would be interesting to evaluate the effect of adenosine receptor antagonists on porcine neutrophils to determine if the presence of the antagonist would alter the effect of the *S. suis* suppressive factor.

The results presented here suggest that *S. suis* releases a molecule composed of purines and other bases and/or nucleosides that inhibits neutrophil function. The exact nature of this molecule, its mechanism of action and its contribution to *S. suis* virulence remains to be determined. A clear understanding of
the molecule and how it functions may help in better understanding the pathogenesis of *S. suis*.

In order to determine the exact structure of this molecule several things must be done. A large quantity of purified sample must be prepared so that reliable repeatable testing can be done. Determination of which purines, pyrimidines, nucleotides and nucleosides are present can be done by comparing elution times through the C18 column of the hydrolyzed unknown fractions to prepared standards. MALDI can be used to confirm mass of the unknowns and compared to MALDI results of the standards. The purified suppressive factor should be analyzed for carbohydrate, e.g., ribose or deoxyribose and nucleotide sequencing should also be done. The results of amino acid analysis have been confusing due to interference by the guanine in the procedure. An elemental analysis of the carbon to nitrogen ratio would help to clarify the presence or absence of amino acids. These procedures should provide much needed information regarding the complete composition of this molecule.

**Acknowledgements**

The authors wish to thank the Iowa State Protein Facility for technical assistance and consultations, Dr. Kim Brogden for assistance with RP-HPLC fractionation of the supernatant and for valuable advice, and Dr. Dagmar Frank and Mr. Tom Skadow for assistance with the neutrophil assays. This work was supported in part by the Iowa Livestock Health Advisory Council.
References


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Table 1. Effect of unfractionated *S. suis* metabolic supernatant on porcine PMN function, expressed as a mean (± SEM) percent of the value for the PBS control.

<table>
<thead>
<tr>
<th>Percent of PBS control</th>
<th>n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodination</td>
<td>48.7 ± 1.1</td>
</tr>
<tr>
<td>Cytochrome C reduction</td>
<td>91.7 ± 7.1</td>
</tr>
<tr>
<td><em>S. aureus</em> ingestion</td>
<td>95.0 ± 4.7</td>
</tr>
<tr>
<td>Migration under agarose</td>
<td>111.7 ± 7.6</td>
</tr>
</tbody>
</table>

Table 2. Effect of RP-HPLC fractions shown in Figure 1 on iodination activity of porcine PMNs. Expressed as a mean (± SEM) percent of the value for the PBS control.

<table>
<thead>
<tr>
<th>RP-HPLC fractions of the 1000 da filtrate</th>
<th>Percent of PBS control</th>
<th>n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 24</td>
<td>88.3 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Fraction 25</td>
<td>89.0 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Fraction 29</td>
<td>91.7 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>Fraction 32</td>
<td>53.0 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Fraction 34</td>
<td>89.7 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>Fraction 40</td>
<td>94.0 ± 10.0</td>
<td></td>
</tr>
<tr>
<td>Fraction 41</td>
<td>97.7 ± 5.0</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. RP-HPLC chromatograph of 1000 dalton filtrate from *S. suis* metabolic supernatant (a representative tracing).
Figure 2. RP-HPLC of hydrolyzed fraction 32 (Figure 1). The guanine standard is superimposed.
CHAPTER 5. GENERAL CONCLUSIONS

Summary of Results

The studies presented in this dissertation address two aspects of the pathogenesis of S. suis disease. The first aspect is experimental reproduction of the clinical disease and lesions, and the second aspect is the interaction of S. suis and neutrophils. The two are related in that in order to cause clinical disease and lesions the S. suis must evade destruction by neutrophils.

Previously published S. suis challenge models used intravenous inoculation of pigs, intranasal inoculation of S. suis to pigs infected with another pathogen, and intraperitoneal inoculation of mice as a model for pigs. In order to evaluate potential virulence factors, vaccine candidates and treatment regimes, an intranasal challenge model without co-infection is desirable. The objective of the experiments in the first part of this dissertation was to develop a S. suis challenge model that mimicked route of exposure, clinical syndrome, and lesions induced in the natural disease. Reports indicated that S. suis administered experimentally via the intranasal route to healthy pigs would not consistently produce disease. It was hypothesized that several factors may be required to have a successful intranasal experimental challenge model. These factors included: irritation of the nasal mucosa prior to exposure to S. suis, aerosolization of the bacteria instead of intranasal inoculation, a high inoculum titer, a challenge bacteria that was directly from an infected pig brain with as little in vitro passaging as possible, and
administration of the challenge inoculum in the growth media because it may contain important virulence factors.

A set of three experiments was performed to develop the intranasal *S. suis* challenge model. All three experiments used a *S. suis* serotype 2 isolate that was obtained by aseptically harvesting the brain/meninges of a pig showing signs of central nervous system disease. The first experiment examined aerosolized *S. suis* with or without pre-exposure to the nasal irritant (ammonia) and also compared the disease induced by aerosolized *S. suis* and intranasally inoculated *S. suis* in pigs exposed to aerosolized ammonia. In this experiment at least 80% of the pigs in each group had some sign of infection. However, only 50% of the pigs that received aerosolized *S. suis* had the bacteria isolated from an internal tissue. There was no difference between the pigs that received the aerosolized *S. suis* following exposure to ammonia and the pigs that were exposed to aerosolized *S. suis* only. Therefore, the disease induced by aerosolized *S. suis* was not enhanced by pre-exposure to ammonia indicating that nasal mucosal irritation was not required. There was a difference in the disease induced between the pigs that were exposed to the ammonia and then challenged with aerosolized *S. suis* and the pigs that were exposed to ammonia and then challenged with *S. suis* by intranasal inoculation. More of the pigs that were exposed by intranasal inoculation had clinical disease, the disease was more severe (higher mortality) and *S. suis* was isolated from more tissues. The role of exposure to ammonia in these groups was not evaluated.

A second experiment was designed to evaluate the role of the ammonia when the *S. suis* was administered by intranasal inoculation. The results of this
experiment indicated that the number of pigs showing clinical disease, the bacterial isolation, and mortality following exposure to *S. suis* by intranasal inoculation was not influenced by pre-exposure to ammonia. The only difference between the two groups was that the pigs pre-exposed to the ammonia had a more rapid onset of the disease.

The intranasal challenge model was used successfully to evaluate the avirulent *S. porcinus* vaccine as a candidate for protection against *S. suis*-induced disease. The vaccine reduced the incidence of mortality, recovery of *S. suis* and clinical signs after *S. suis* challenge; however, the differences were not statistically significant. Therefore, further development of the *S. porcinus* vaccine was not pursued.

The third experiment was designed to begin to determine why the challenge model was successful. The objective of the experiment was to determine if the intranasal challenge induced disease because the stock culture was a brain/meninges homogenate and the challenge inoculum was prepared without further passaging. The stock culture was passaged 20 times on blood agar plates (P20) and then a challenge inoculum was prepared in the same manner as the original challenge inoculum (P1). Both P1 and P20 caused signs of clinical disease in ≥ 80% of the pigs. *S. suis* was isolated from internal tissues of 80% (P1) and 70% (P20) of the pigs, and there was 40% mortality in the P1 group and 60% mortality in the P20 group. There was no significant difference (p>0.05) between these two groups for the parameters measured. Therefore, in vitro passaging 20 times did not
decrease the virulence of the bacteria. This experiment confirmed the reproducibility of this model.

The reason the *S. suis* challenge model described in this dissertation is successful in mimicking the natural disease is still unclear. One possibility is that the bacteria are administered in the growth media. The growth media may contain important virulence factors that allow the bacteria to evade immune destruction and establish infection that causes clinical disease.

The second part of this dissertation partially addresses the hypothesis that the growth media contains important virulence factors. The *S. suis* serotype 2 isolate was grown in vitro in Todd Hewitt Broth and then washed and incubated in phosphate buffered saline (PBS) for 2 hours. The supernatant was examined for its influence on porcine neutrophil function. The supernatant suppressed the myeloperoxidase-\( \text{H}_2\text{O}_2 \)-halide activity of neutrophils to 49% of a PBS control, it did not suppress or only mildly suppressed neutrophil migration under agarose, ingestion of *Staphylococcus aureus* or oxidative metabolism when compared to PBS control. The suppressive activity in the supernatant was monitored using the iodination assay that measures the MPO-\( \text{H}_2\text{O}_2 \)-halide activity of neutrophils. The suppressive activity in the supernatant was stable to heating, was not present if the supernatant was prepared at 4°C instead of 37°C and was not present in a *S. suis* capsular preparation. The suppressive activity passed through a 1000 dalton cut-off filter. The filtrate was then fractionated by C-18 reverse phase-high performance liquid chromatography (RP-HPLC) and the suppressive activity was recovered from fraction 32. Fraction 32 was then evaluated for composition and found to contain
guanine and two unknown components that may be other bases and/or nucleosides. Therefore, *S. suis* releases a small molecular weight molecule that decreases neutrophil function which may contribute to the virulence of *S. suis*.

**Application to the Problem**

The challenge model developed is an important tool for furthering the understanding of *S. suis* pathogenesis. This model is important for the evaluation of potential virulence factors, vaccine candidates and treatment regimes. It is unique from other *S. suis* challenge models that have been reported in that the *S. suis* is administered via the natural route of infection, it is the only pathogen administered and the clinical disease and lesions produced are similar to those that are observed in the field. This challenge model has been used in two studies evaluating various prevention and therapeutic strategies for *S. suis*. Manuscripts reporting the results of these studies are in preparation.

The interaction between neutrophils and *S. suis* is important. It is thought that virulent isolates of *S. suis* are able to survive in phagocytic cells and avirulent isolates are not able to survive. The mechanism that allows the streptococcus to do this is unknown. Capsule thickness shows some correlation to increased resistance to neutrophil killing; however, the resistance to neutrophil killing was not always correlated with a thickened capsule. Therefore, there are other factors that may allow the virulent isolates to survive and resist phagocytic killing. The research presented in this dissertation describes a molecule that is released by *S. suis* into
saline that decreases the neutrophil MPO-H₂O₂-halide activity. This factor may be important in the virulence of *S. suis* and in its interaction with neutrophils in vivo.

**Recommendations for Future Research**

Further research is needed to fully characterize the challenge model. Understanding why this model successfully reproduces the disease may provide information about the pathogenesis of *S. suis*. Some possible reasons why the challenge model worked include: the high titer of the challenge inoculum, administration of the *S. suis* in growth media without washing, the way the inoculum was grown, unique attributes of the isolate, or age of the pigs challenged. A variation of this model was used in a porcine respiratory and reproductive virus and *S. suis* coinfection model. We diluted the standard *S. suis* challenge inoculum 1:10 in a balanced salt solution, this not only decreased the titer but also diluted the growth media. The diluted inocula only caused clinical signs and mortality in 14% of the animals. This indicates that the titer and/or administration in undiluted growth media are important for this challenge model to induce disease. It would be interesting to test other *S. suis* isolates that were originally recovered from cases of meningitis, and under different challenge conditions did not induce disease, using identical procedures to those reported here.

A better understanding of the interaction of *S. suis* and neutrophils is needed to develop better preventive and therapeutic regimes. Identification of the exact structure and mechanism of action of the neutrophil suppressive factor described in
this dissertation would help in understanding that interaction. In addition, determining the role this molecule plays in vivo would also be important in understanding its significance in the pathogenesis of *S. suis*.

References


