Molecular and Functional Characterization of Key *Staphylococcus pseudintermedius* Virulence Proteins: Paving the Way for Vaccine Development

A Dissertation Presented for the

Doctor of Philosophy

Degree

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DEDICATION

I dedicate my work to all of my family and my friends.

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ABSTRACT

Staphylococcus pseudintermedius is the primary cause of canine pyoderma. The vast majority of *S. pseudintermedius* are resistant to all antimicrobials available to veterinarians. Accordingly, there is a need for alternative approaches to control staphylococcal infections, such as vaccines. Development of vaccines to control staphylococcal infections is a high priority, however there are no licensed *S. pseudintermedius* vaccines. This is likely due to a lack of information about *S. pseudintermedius* protein functions, surface accessibility and epitope conservation. Effective staphylococcal defenses are rooted in the ability of the bacteria to neutralize and/or destroy important components of their host's defenses. The objective of this study was to identify and direct the immune response against the most important antigen targets. This was achieved using three strategies

(a) Identification and understanding the roles of protein A, leuxotoxin-I, SpEX [*S. pseudintermedius* Exotoxin] and 5' nucleotidase in *S. pseudintermedius* pathogenesis and host immune response evasion.

(b) Attenuation of these proteins to make them safe without eliminating epitopes that induce a protective immune response.

(c) Evaluation the neutralizing effect of specific antibodies developed in clinically health dogs against these proteins.

In this study, a conserved *S. pseudintermedius* protein A, leuxotoxin-I, SpEX [*S. pseudintermedius* Exotoxin] and 5' nucleotidase *in vitro* were characterized and identified as a critical virulence factors for immune suppression. *S. pseudintermedius* protein A binds antibody on bacterial surface preventing destruction of bacteria and serves as superantigen that destroys B cells and blocks host antibody response. Leukotoxin I kill host leukocytes preventing innate and adaptive immune response. *S. pseudintermedius* Exotoxin kills host leukocytes preventing innate and adaptive immune response and neutralizes complement. 5' nucleotidase converts host adenosine tri and mono phosphate to adenosine which inhibits phagocytosis and bacterial destruction and clearance by neutrophils.

Attenuated immunogenic recombinant proteins expressed from synthetic genes altered to produce the attenuated proteins. Clinically healthy dogs were used to develop neutralizing antibodies against the injected attenuated proteins and their native homolog. By neutralizing surface-bound and extracellular proteins responsible for host immunosuppression, these mutant proteins may serve as important components of a multivalent vaccine for prophylaxis of *S. pseudintermedius* infections.

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
AdsA	Adenosine synthase A
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCPFTs	Bicomponent pore-forming toxins
bp	Base pair
°C	Degree Celsius
cc	Cubic centimeter
C5	Complement component 5
CCR5	Chemokine receptor type 5
CD	Cluster of differentiation
CDS	Coding DNA sequence
CXCR	Chemokine receptor
ClfA	Clumping factor A
CP5	Capsular polysaccharide 5
CP8	Capsular polysaccharide 8
CNS	Coagulase-negative Staphylococci
CPS	Coagulase-positive Staphylococci
CWA	Cell wall-associated proteins
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco’s modified Eagle Medium

DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix proteins
EDTA	Ethylenediaminetetraaceticacid
ELISA	Enzyme-linked Immunosorbent Assay
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FITC	Fluorescein isothiocyanate
FnBP	Fibronectin binding protein
g	Gram
h	Hour
HIg	gamma hemolysin
HRP	Horse-radish peroxidase
IACUC	Institutional Animal Care and Use Committee
IgBD	Immunoglobulin binding domains
IsdB	Iron-regulated surface determinant B
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA	Immunoglobulin A
lPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
kDA	Kilodalton

LB	Luria-Bertani broth
Luk	Leukotoxin
LPXTG	motif in cell wall-associated proteins with the amino acid composition
M	Milli
mecA	Gene encoding methicillin resistance
min	Minute
mM	Milli-mol
mm	Millimeter
MS	Mass spectrometry
MSA	Multiple sequence alignment
MALDI-MS	Matrix assisted LASER desorption ionization- mass spectrometry
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *S. pseudintermedius*
MRA	Microbiology Resource Announcements
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
MSSP	Methicillin-susceptible *S. pseudintermedius*
µ	Micro
MFI	Mean Fluorescent Intensity
ng	Nano gram
ns	no significant difference
NPPc	Non-professional phagocytic cells

ORF Open reading frame

OB Oligosaccharide/oligonucleotide binding

OD Optical density

OPK Opsonophagocytic killing

PBS Phosphate buffered saline

PBS-T Phosphate buffered saline containing 0.05% polyethylene glycol sorbitan monolaurate (Tween 20)

PE Phycoerythrin

PCR Polymerase Chain Reaction

PYR Pyrrolidonyl aryalamidase

PMNs Polymorphonuclear Neutrophils

PBMC Peripheral blood mononuclear cells

pSORT Predetermined cellular location

SAg Superantigen

SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SIG Staphylococcus intermedius group

SIET *Staphylococcus intermedius* exfoliative toxin

STs Sequence Types

Srt A Sortase A

Spa Staphylococcal Protein A

Sps *S. pseudintermedius* surface proteins

SpAdsA *S. pseudintermedius* Adenosine synthase

SpEX *S. pseudintermedius* EXtoxin

SSL	Staphylococcal superantigen-like
TMB	Tetramethylbenzidine
TSB	Trypticase soy broth
UK	United Kingdom
VH3	Variable heavy
AA	Amino Acids
WGS	Whole Genome sequencing
x g	Times gravity
α	alpha
β	beta

CHAPTER 1
INTRODUCTION

Introduction and Review of Literature

Staphylococci are common among commensal bacteria that reside on human skin and mucous membranes and various species of animals (1). Differentiating between coagulase-positive [CPS] and coagulase-negative staphylococci [CNS] is useful in terms of their medical significance. CPS encompasses *S. aureus, S. pseudintermedius, S. intermedius, S. schleiferi subsp. coagulans, S. hyicus, S. lutrae, S. cornubiensis* and *S. delphini* and these bacteria were generally considered capable of causing opportunistic infections in humans and different species of animals (2). CPS-produced bacterial coagulase is a virulence factor as the enzyme allows the bacteria to induce fibrinogen conversion to fibrinopeptides and self-polymerizing fibrin (2). It is regarded as an important first step in the attachment and adhesion of CPS within the invaded body, which promotes staphylococcal growth during the early stages of infection. The coagulum later protects the staphylococci from defensive cell activities. Once infection is established the expression of another enzyme (staphylokinase) allows the bacteria to escape the fibrin-mediated wall that is self-induced. Subsequently, other factors associated with virulence, such as hyaluronidase (spreading factor), lipase, collagenase, protease, nuclease, and urease may all play additional roles in staphylococcal infection pathogenesis (3).

Staphylococcus intermedius group [SIG]

Until 2005, *Staphylococcus intermedius* was thought to be the causative agent of canine skin infections based on phenotypic traits. However, in recent years, studying the bacterial genome using molecular typing methods led to the discovery of a novel species, *S. pseudintermedius*, in 2005 as the causative agent of canine pyoderma and other skin infections [7] and *Staphylococcus cornubiensis* isolated from a human skin infection in Cornwall, United Kingdom (4).

Phylogenetic analysis grouped *S. intermedius, S. pseudintermedius, S. cornubiensis* and *S. delphini* in the Staphylococcus intermedius group (SIG) where *S. cornubiensis* is genomically relative to *S. intermedius* with a core genome nucleotide identity of 90.2 % (4) and phenotypically similar to *S. pseudintermedius*. Traditional phenotypic diagnostic tests are not reliable for distinct differentiation between SIG isolates and also from other

staphylococci. There is a combination of biochemical tests that can be used to differentiate between different species within SIG group (**Table 1.1)**.

Pyrrolidonyl aryalamidase (PYR) can effectively separate the SIG members (PYR positive) from *S. aureus* (PYR negative) (5).

Knowledge of the host range aids in the clinical identification of *S. pseudintermedius* in a diagnostic setting where *S. intermedius* is more or less exclusively isolated from pigeons and *S. delphini* has been reported to be isolated from dolphins, horses, cattle, mink, human and not associated with infections in dogs. Due to their similar biochemistry, molecular typing is the most accurate method to identify the species within the SIG.

Pathogenesis of *S. pseudintermedius*

S. pseudintermedius is an opportunistic pathogen. It causes disease when the immune system of the host is compromised or an external skin barrier breach occurs, such as wounds and surgical procedures or other factors that predispose the host to atopic dermatitis. *S. pseudintermedius* is frequently isolated from the nares, mouth, forehead, groin and anal region of healthy dogs and cats as a common inhabitant of the skin and mucosa (6).

Recent advances in genome sequencing technology and bioinformatics have added a wealth of information to our knowledge about proteins produced by *S. pseudintermedius*. More than 170 complete genome sequences of *S. pseudintermedius* have recently been reported to help analyze the potential for virulence and special host adaptation of this versatile opportunistic pathogen (7-14). ED99 and HKU10-03 sequence analysis resulted in the identification of numerous putative virulence factors including luk-I, exotoxins, protein A and exoenzymes (13, 14).

S. pseudintermedius is a CPS species with numerous virulence factors (**Figure 1.1)**, some of which are similar to those described for *S. aureus* (15) such as proteases, coagulase as well as proteins associated with the surface such as 5’nucleotidase and protein A. *S. pseudintermedius* can produce a variety of toxins including leukotoxins, enterotoxins and other toxins that cause substantial damage to affected host tissue (16, 17).

Table 1.1 phenotypic characteristics of SIG isolates (4).

Characteristic	*S. cornubiensis* NW1T	*S. intermedius* DSM 20373	*S. pseudintermedius* DSM 21284	*S. delphini* DSM20771
Pigment	−	−	−	−
Coagulase	+	+	+	+
Clumping factor	−	−	−	−
DNase	+	+	+	−
β-Haemolysin	+	+	+	+
Mannitol fermentation	+	+	−	+
Acetoin	−	−	−	−
Pyrrolidonyl aryalamidase*	+	+	+	+
Maltose*	−	+	+	+
d-Galactose*	+	+	+	+
Trehalose*	+	+	+	−
Sucrose*	+	+	+	+

*Data based on VITEK2 GP card (bioMérieux)(4).

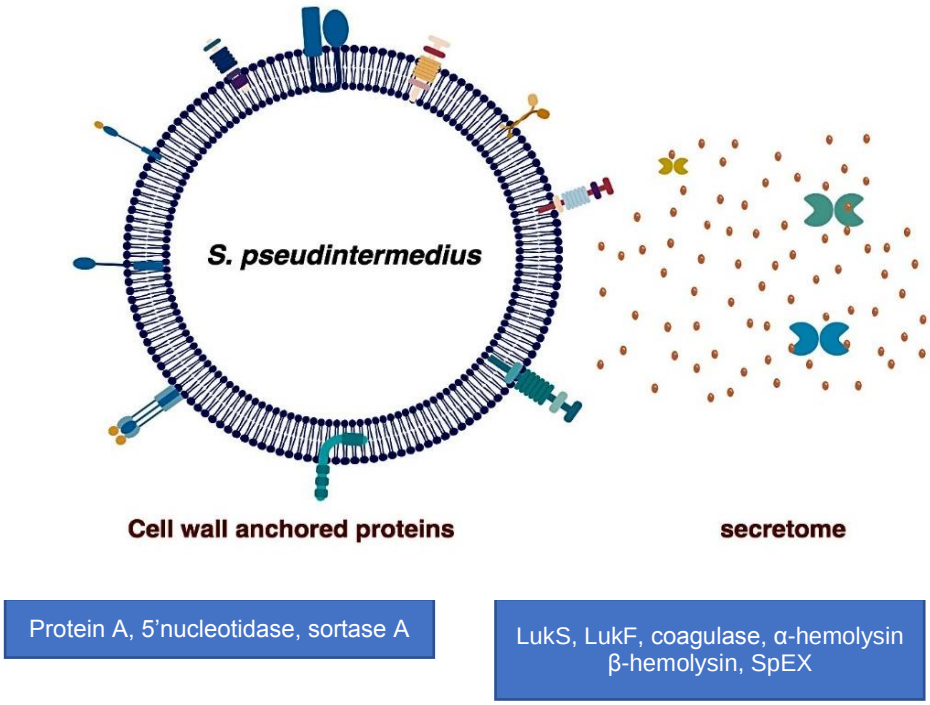


Figure 1.1 Schematic diagram for *S. pseudintermedius* cell wall and secreted virulence proteins.

S. pseudintermedius causes hemolysis of rabbit erythrocytes and hot-cold hemolysis of sheep erythrocytes through α-hemolysin and β-hemolysin (18). The β-hemolysin has high affinity and is responsible for the characteristic partial zone of hemolysis observed in sheep blood agars around colonies (18). It also produces

a two-component leukotoxin, luk-I, encoded on polymorphonuclear cells by two co-transcribed genes, lukS and lukF (17). Luk-I is associated with the most prevalent clonal population, ST71 in Europe, occurring especially methicillin-resistant isolates.

S. pseudintermedius is also capable of binding to fibrinogen, fibronectin and cytokeratin using surface molecules that act as microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (15). Proteins associated with the cell wall (CWA), such as microbial surface components that recognize adhesive matrix molecules (MSCRAMMs), play an essential role in bacterial attachment to the extracellular matrix of the host. In each bacterial species, the repertoire of MSCRAMMs contributes to host adaptation and survival. *S. pseudintermedius* CWA proteins have been named as 'Sps'

(*S. pseudintermedius* surface proteins). In particular, at least 19 CWA proteins are known as SpsA through SpsR in isolate ED99 (19, 20).

Sortase A is a cysteine transpeptidase enzyme that most Gram-positive bacteria produce. During pathogenesis, surface proteins play a crucial role in Gram-positive bacteria. The main function of sortase A is to anchor surface proteins with a C-terminal LPXTG motif onto the peptidoglycan cell wall. Sortase A anchored proteins act as nutrient transporters, adhesins, internes, blood clotters and immune evasion factors (21). *S. aureus* lacking sortase cannot assemble LPXTG proteins on their surfaces and cannot form abscess lesions in organs or cause lethal bacteremia in animal models of infection (22). Sortase A is the prototypical enzyme for class A families and has been studied extensively. In Firmicutes, Sortase A is present, and responsible for the anchoring of proteins mediating bacterial adhesion.

In the genome sequences of different *S. pseudintermedius* isolates that represent the main clonal populations in the United States, Asia and Europe, a putative srtA gene was identified. Functional domains were identified on the predicted protein sequence. *S. pseudintermedius* Sortase A has amino acid similarity of 57% with *S. aureus* Sortase A.

Protein A

A number of potential virulence factors, such as cell wall anchored proteins (CWA), secreted enzymes and toxins, may explain the fitness of staphylococci .These virulence factors have been more thoroughly investigated and characterized in *S. aureus* than in *S. pseudintermedius*, although the majority have been shown to have similar features. This is the case of the Staphylococcal protein A (SpA), a 40-60 kDA protein in the most prevalent group of CWA proteins. In *S. aureus*, SpA includes five repeated domains (E, D, A, B and C) **(Figure 1.2)**, each of which binds the Fc region immunoglobulin (Ig) G with high affinity and the variable heavy VH3 subclass Fab region of Ig. Practically all clinical *S. aureus* isolates express and secrete protein A during the exponential growth phase. Because of its binding activities on human and animal Igs, protein A is immunosuppressive. Protein A binding to Fc region of IgG blocks opsonophagocytic killing (OPK), while Fab binding and IgM cross-linking promotes B cell superantigen activity. In particular, B-cell receptor SpA crosslinking causes an expanding lymphocyte

population to proliferate and to collapse while undergoing apoptosis. Together with century-long studies in staphylococcal vaccines, it was recently shown that immunization with nontoxigenic protein A (SpA_{KKAA}) allowed infected Guinean pigs to trigger an anti- *S. aureus* protective antibody response.

In view of these considerations, Balachandran et al investigated the common characteristics between *S. aureus* and *S. pseudintermedius* protein A to understand if *S. pseudintermedius* protein A may be a vaccine candidate. There is little known about *S. pseudintermedius* protein A so far. Indeed, Moodley et al. (23) initially described a putative *spa* gene in 2009; subsequently, sequencing of the entire genome of *S. pseudintermedius* ED99 revealed that two genes had an orthologue conformation with *S. aureus spa*, *spsP* and *spsQ*. The SpsP protein is composed of a predicted N-terminal sequence of 33 amino acids and three IgG-binding domains. The C-terminal region has a predicted X region with a sequence similarity of 63 percent to that of *S. aureus*. The *spsQ* gene, on the other hand, encodes a protein consisting of 462 amino acids with a predicted N-terminal sequence of 33 amino acids, followed by a repeating region comprising 4 IgG-binding domains.

The C-terminal region has a predicted X-region that includes a repeat sequence (Xr) of 77 amino acids and a constant region (Xc) with 70% similarity to *S. aureus* X-region.

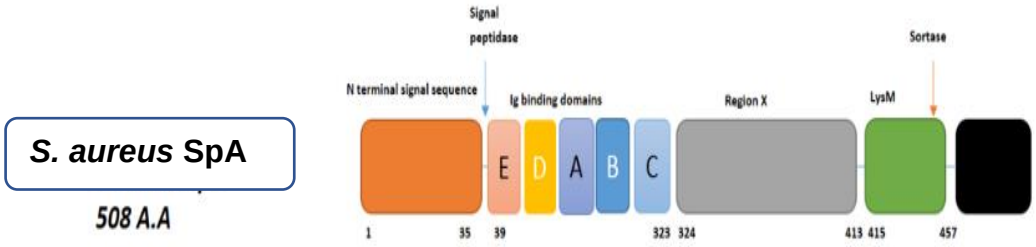


Figure 1.2 Schematic diagram for *Staphylococcus aureus* protein A

Riley et al. recently reported (9) that the full-length *spsQ* gene was consistently present in clinical isolates of *S. aureus*, while *spsP* gene was less frequently portrayed by *S. pseudintermedius*.

A total of 18 clinical *S. pseudintermedius* isolates with various lineages were examined by Balachandran et al. (21) and she showed that the *spsQ* gene was present in all isolates, as already observed, but its expression level differed between STs and the protein A secreted in supernatant. Compared with other sequence types, the difference was found to be significant for ST71 compared with ST68, and also for these two clones. These results provide further information on the global success of the two lines, which are a major challenge in the treatment of MRSP infections nowadays. In general data about *S. pseudintermedius* virulence is scarce and there is a need for continuous efforts to better understand the pathogenicity of this organism. The relationship between dogs and their owners has dramatically changed during the last decade. Microorganisms are increasingly transmitted between people and their dogs (24). As was shown by the MDR ST71, which has been isolated at a higher frequency both in humans and animals. The risk of MRSP infection is unclear in humans however, evidence is given of potential long-term human MRSP carriage due to the particular adaptive ability of certain MRSP lines, such as ST71 and ST68, to the human host.

S. pseudintermedius SpsQ is a virulence factor that permits the bacterium to evade the immune response. SpsQ can bind IgG through its Fc regions to protect staphylococci from opsonophagocytosis **(Figure 1.3)**. Thus, *S. pseudintermedius* protein A enables bacteria to escape opsonization and phagocytosis which results in persistent diseases. Phagocytosis assays performed by Balachandran (21) also confirmed these results. On the other hand, several steps towards the immunological aspects of *S. aureus* SpA have been made. Modification of the immunoglobulin function by SpA may have impede the elicitation of antibody-responsive reactions to bacterial envelope elements (CP5/CP8, ClfA, and IsdB). Virulence contributions of protein A depend on its binding to both Fc and Fab domains of IgG **(Figure 1.3)**. Incremental defects in systemic infection pathogenesis were reported in *S. aureus* spa_{KK} and spa_{AA} variants. In addition, contributions of

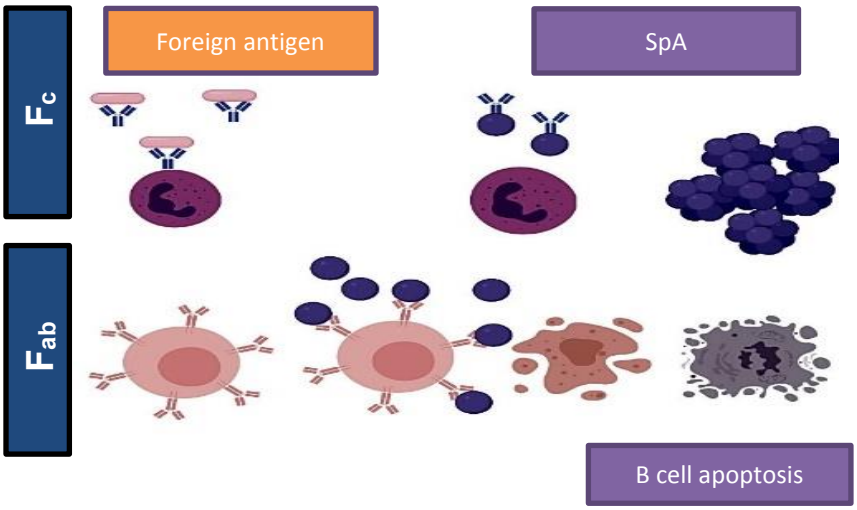


Figure 1.3 Mechanisms of SpA-mediated immune evasion

S. aureus protein A to virulence were not present in mice lacking both mature B cells and their antibody products.

In contrast to wild-type staphylococci infection, antibodies that neutralize SpA had been elicited by the immunization of mice or rabbits with an engineered SpA_{KKAA} in which 20 amino acid residues, essential for their combination with IgG Fc and Fab were replaced. SpA_{KKAA}–derived polyclonal antibodies protect the B cells from SpA, stimulate staphylococcal phagocytosis and promote humoral immune reactions to a wide range of antigens. In addition, studies using SpA_{KKAA} monoclonal antibodies (MAb) have confirmed these observations (25).

leukocidin

The first PVL positive MRSA was seen at the end of 1990 and in recent years it has been distributed globally (26). The role of PVL in *S. aureus* virulence and pathogenicity are under investigation. The pathogenicity of *S. aureus* is increased by Panton Valentine leukocidin through necrosis, acceleration of apoptosis and killing of polymorphonuclear and mononuclear cells (27). Some studies have shown, however, that PVL is not associated with organism virulence by showing improved clinical outcomes from skin and

soft tissue infections (28, 29). The role of PVL in clinical results is therefore still under discussion. The reason for the clinical results in these studies could be determined by the efficacy of antibiotic therapy. The marker of community-acquired MRSA, responsible for soft tissue and deep dermal infections, is Panton Valentine leucocidin (30, 31). However, the overall PVL scenario varies between MRSA isolates. The increasing prevalence of PVL among MRSA isolates is reported from various countries (32, 33). The prevalence of PVL among MRSA and MSSA (MRSA: 85.1 and MSSA: 48.8 percent) has been reported in India, which shows a higher prevalence of PVL in MRSA (34). The PVL prevalence in other parts of the world has decreased (35-37), reflecting a significant variation in geographical location and population prevalence, which included 5% in France, 4.7% in Great Britain, 8.1% in Saudi Arabia and 14.3% in Bangladesh.

A key feature of *S. aureus* pathogenesis is its capacity to secrete a wide range of immune evasion factors. These include pore-forming toxins that are of interest in developing new therapeutic approaches (38). Toxins that form pores are virulence factors that are expressed as monomeric proteins that are water-soluble. They assemble on the membranes of the target cells to form a pore after the receptors of their hosts have been recognized (39). The pore-forming toxins can be classified into two families based on the secondary structure of the transmembrane region of the pore structure: the -helical family and the -barrel family. The β-barrel family consists of one member of the pore-forming toxin single-component, αHL, and three members of the bicomponent pore-forming toxin, γ-hemolysin (γ-HL), Panton–Valentine leukocidin (PVL) and leukocidin (Luk) (40). Seven known bicomponent leucocides have been found to date: LukSF-PV, HlgAB, HlgCB, LukAB/HG, LukED, LukPQ and LukMF (41).

There are two separate water-soluble subunits of the bicomponent pore-forming toxins, one of class S (slowly-eluted component of PVL) and one of class F (fast-eluted component of PVL). Recently, the list of host molecules which interact with the bicomponent pore forming toxins has also included a number of protein receptors, which greatly enhances the understanding of specific cell targeting by *S. aureus* (42).

Indeed, for most leukocidins, certain cell membrane lipids are known to bind and even facilitate the prepore-to-pore transition (43, 44). Typically, the S subunit is involved in

targeting the specific cell type through interaction with surface receptors and recruiting the other toxin subunits, resulting in oligomerization and octameric prepore formation. The prepore consists of alternating S and F subunits and is capable of assembling a -barrel pore domain in the cell membrane, producing the mature pore that ultimately causes osmotic lysis to cause cell death (45).

The complex of mushroom-shaped pore is divided into three domains resembling the domains of soluble monomers: cap, rim, and stem. The cap is made up of sandwich and latch domain of each protomer. The rim domain is an open-face sandwich that extends the cap domain below. The rim is important for the lipid bilayer interaction and the interaction with specific receptors of the membrane. The stem domain is involved in the transmembrane-barrel formation in each protomer, which ultimately perforates the membrane. Secreted water - soluble monomers reveal a similar overall protomer structure except for the stem region, which is more compact than three antiparallel –strands stacked with cap domain -strands (46-49).

LukED is one of the key virulence factors that plays a vital role in *S. aureus* bloodstream infections as shown by the dramatic attenuation of an isogenic highly virulent staphylococcal strain with LukED deleted in animal models (50, 51). LukED is also the only highly neutrophil - defeating leukotoxin (50). LukED targets a wide range of host cell that has been partially clarified by recent identification of its binding partners (CCR5, CXCR1 and CXCR2)(50).

LukED can target both innate and adaptive immunities by binding these three cell receptors. Indeed, among the sequenced *S. aureus*, LukE and LukD display almost no sequence diversity (52). In addition, the association of LukED with staphylococcal bullous impetigo and post-antibiotic diarrhea has been shown (53). These findings identify LukED as a target for alternative and more effective staphylococcal infection therapy.

In an earlier study, mutants of *S. aureus* LukS-PV and LukF-PV were rationally designed to be deficient in oligomerization by Karauzum et al.(54) Testing mutant versions of LukS - PV with a series of amino acid substitutions, they found LukS - mut9 with T28F / K97A / S209A to be highly immunogenic and non - cytototoxic when mixed with LukF - PV [18]. LukS -PV - raised rabbit immunoglobulin reduced the cytotoxic effect of gamma

hemolysin A and B subunits, gamma hemolysin C and B subunits and LukE and LukD, and non-canonical pairs gamma hemolysin B subunit and LukE, gamma hemolysin C subunit and LukD and gamma hemolysin A subunit and LukD (55).

S. pseudintermedius also produces Luk-I leucotoxin, which is very similar to *S. aureus* Panton - Valentine leucocidin (PVL) (56, 57). *S. pseudintermedius* produces a two - component leukotoxin, Luk-I, composed of secreted LukS-I and LukF-I proteins that induce cell lysis.

Ruzauskas et al. (58) isolated *S. pseudintermedius* from dogs with various diseases and therefore found genes that encode pathogenic factors. A high frequency (68.8 %) of luk-I and/or siet genes was detected in *S. pseudintermedius* isolated in Lithuania. It is interesting to note that all isolates that harbored both lukF and lukS genes also harbored the siet gene ($p<0.01$), but not vice versa. Such a statistical relationship had not been detected before to our knowledge. In the study of the association between the presences of siet gene in isolates from skin infections compared to isolates from other types of infection, statistically significant results were obtained (58).

Siet gene is primarily found in isolates from skin infections (59), this is consistent with the fact that exfoliative toxin is a key virulence factor in *S. pseudintermedius* causing canine pyoderma. The luk-I genes have been found in various *S. pseudintermedius* strains regardless of the source of isolation. Luk-I demonstrates strong leucotoxicity towards different polymorphonuclear cells (57). By suppressing its cellular immunity, it could be responsible for the host invasion and could be produced by different *S. pseudintermedius* strains.

Maali et al. (60) tested the effect of Luk-I on non-professional phagocytic cells (NPPc) and no cytotoxic activity was observed with recombinant Luk-I toxin against NPPc compared to that found for PMN cells. In response to this issue, he conducted an in vitro trial with promyelocytic U937 cells stably transfected to C5a and CXCR2, which are the PVL and LukED leucotoxin target receptors. The results indicated that, as previously reported for *S. aureus* LukED, Luk-I induces cell death through the CXCR2 receptor specifically present on immune cells (PMNs, macrophage), explaining the lack of NPPc

cytotoxicity. This feature is probably related to the high Luk-I and LukED homology (61). Focused on humans as well as dogs, this receptor is also highly homologous (75 %) (62). Innate immunity is important in the defense against staphylococcal and neutrophil migration and represents the first step of the acute inflammatory response toward infection. This process starts with neutrophils binding to inflamed endothelium, migration toward a site of inflammation, followed by phagocytosis of infectious organisms. It is largely dependent on integrin molecules in the neutrophil membrane binding to endothelial receptors (63) and phagocytosis is enhanced by opsinins.

S. pseudintermedius **exotoxin protein**

Staphylococcal SSLs are two- domain secreted proteins with an average size of 25 kDa (64, 65). The first domain, located at the N-terminus, displays an oligosaccharide/oligonucleotide binding (OB) fold forming a β- barrel. The second domain possess a β-grasp motif that consists of a twisted β-sheet of four to five antiparallel strands, located at the C-terminus. The two domains are separated by a structurally conserved α-helix. Tuffs et al., (66) showed that staphylococcal enterotoxin-like toxin (SEIX) secreted by *S. aureus* shares their domain structure with SSLs. SSLs target innate immunity components through different mechanisms such as complement binding but do not bind to T cell receptors or the major histocompatibility complex. SEIX exerts the same function, however, it exhibits a superantigen effect via its oligosaccharide/oligonucleotide binding (OB) fold on T cells (66, 67).

In previous studies, SSL4 (67), SSL5 (65) and SSL11 (65), were found to target myeloid cells and interfere with neutrophil migration through their sialated glycans-binding site in the C- terminal β-grasp domain. Chung et al., (68) showed that SSL11 with a single site mutation, T168P, had defective binding compared to native SSL11 and lost its inhibitory effect on neutrophil attachment to P-selectin. The OB fold domain of SSL7 binds to the IgA Fc region while the β-grasp motif adheres tightly to complement component C5 resulting in inhibiting of complement mediated hemolytic activity (65). SSLs are not associated with cytotoxic activity.

S. pseudintermedius 5-nucleotidase

Upon infection or cell damage, high amounts of adenosine triphosphate (ATP), adenosine monophosphate (AMP), adenosine diphosphate (ADP) are released from damaged cells, which serve as mediators of inflammation through purinergic cell surface receptor signaling (69, 70). As a control mechanism to prevent excessive damage to host tissue, adenosine counteracts the effect of ATP by stimulation of adenosine receptors suppressing the pro-inflammatory response. Four adenosine receptors have been described: A1R, A2aR, A2bR and A3R. Neutrophils express all four adenosine receptors (71, 72). They are the most abundant mammalian leukocytes and form an important component of the innate immune response against staphylococcal infection (73, 74).

In mammals, extracellular nucleotide and adenosine concentrations are controlled by nucleotide metabolizing enzymes. In this two-step process, CD39 catalyzes dephosphorylation of ATP and ADP to produce AMP. 5′-nucleotidase (CD73) contains a characteristic two-signature sequence domain and hydrolyzes AMP into adenosine (74, 75). Some bacilli employ adenosine synthase to interfere with the balance between extracellular nucleotides and adenosine by perturbing the adenosine level beyond the physiological range (76, 77). Extracellular 5′-nucleotidases have been identified in *S. aureus* and *Streptococcus suis* type 2 (75, 78) and shown to play an important role in evasion of the innate immune response.

Thammavongsa et al, (75) showed that *S. aureus* can escape phagocytic clearance in blood and identified adenosine synthase A (AdsA), a cell wall–anchored enzyme that converts adenosine mono- and tri-phosphate to adenosine, as a critical virulence factor. Staphylococcal synthesis of adenosine in blood, escape from phagocytic clearance, and subsequent formation of organ abscesses were all found to be dependent on AdsA. They observed further that AdsA-deficient *S. aureus* were cleared more rapidly from the bloodstream of BALB/c mice than wild-type staphylococci, correlating with reduced ability to grow during infection and/or seed abscesses. Furthermore, adenosine attenuates neutrophil antibacterial activity by suppressing the production of superoxide and nitric oxide both of which are integral to the killing of phagocytosed bacteria (72, 75).

S. aureus AdsA has critical residues (Asp127 and His129) for divalent metal cation binding located in the first nucleotidase signature sequence (ILHTNDIHGRL) (75) The

essential amino acids for catalytic activities of AdsA, His196 and Asp199, represent the catalytic Asp-His dyad and are located in the second signature sequence (YDAMAVGNHEFD) (75).

Adenosine synthase activity has not been previously described in *S. pseudintermedius* or any members of the *Staphylococcus intermedius* group.

S. pseudintermedius is the primary cause of canine pyoderma (skin infection). Pyoderma is the most common canine dermatologic disease and the organism is also associated with urinary tract infections, wound and surgical site infections, external ear otitis, abscess formation, mastitis and endocarditis. As many as 30-40% of the *S. pseudintermedius* isolates tested in clinical laboratories in some geographical regions are methicillin-resistant (MRSP). These bacteria are resistant to all β-lactam antibiotics. The vast majority of MRSP are also resistant to other clinically useful antibiotics and an increasing number of MRSP are resistant to all antimicrobials available to veterinarians. Accordingly, there is a need for immunogenic compositions capable of inducing antibodies to, and/or a protective immune response to *S. pseudintermedius*.

S. pseudintermedius **Vaccine Development**

Development of vaccines to control staphylococcal infections is a high priority, however there are no licensed *S. pseudintermedius* vaccines. This is likely due to a lack of information about *S. pseudintermedius* protein functions, surface accessibility and epitope conservation. Information about other species of bacteria, especially staphylococci, is useful in the development of a *S. pseudintermedius* vaccine, however, this species is unique with regard to its disease presentation, genetic structure and composition, host range, and the virulence proteins it produces. Whole bacterial staphylococcal vaccines have not been efficacious. With an estimated greater than 2,000 different proteins produced by each bacterium, it is essential to identify and direct the immune response against the most important antigen targets. However, many of these proteins are potentially harmful to animals and must be attenuated to make them safe without eliminating epitopes that induce a protective immune response. Effective staphylococcal defenses are rooted in the ability of the bacteria to neutralize and/or destroy important components of the immune system of their hosts.

This dissertation is organized in a manuscript format. Each chapter is a manuscript. Chapter 1 is Introduction and literature of review. Chapter 2 characterize recombinant wild-type and nontoxigenic protein A from *S. pseudintermedius* that have been previously published in Virulence Journal. In Chapter 3, a leucocidin produced by *S. pseudintermedius* has been identified and characterized and published in PLOS One Journal. The first whole-genome sequences for *S. pseudintermedius* Type Strain LMG 22219 (=ON 86T = CCUG 49543T) are presented in Chapter 4 and were published in Microbiology Resource Announcements (MRA), an online-only, fully open access journal that publishes articles announcing the availability of any microbiological resource deposited in a repository available to the community. In Chapter 5, SpEX produced by *S. pseudintermedius* has been identified and characterized. In Chapter 6, 5’ nucleotidase produced by *S. pseudintermedius* has been identified and characterized. Conclusion and Recommendations in Chapter 7.

CHAPTER 2
CHARACTERIZATION OF RECOMBINANT WILD-TYPE AND NONTOXIGENIC PROTEIN A FROM *STAPHYLOCOCCUS PSEUDINTERMEDIUS*

A version of this chapter was originally published by Mohamed A. Abouelkhair, David A. Bemis, Stephen A. Kania:

Abouelkhair MA, Bemis DA, Kania SA. 2018. Characterization of recombinant wild-type and nontoxigenic protein A from S. pseudintermedius. Virulence 9:1050-1061

No revisions were made to the original published manuscript beyond general formatting to allow consistency through the dissertation. My contributions to this paper include: (i) selection of isolates (ii) experimental design and set up (iii) experimental testing (iv) data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) writing.

Abstract

S. pseudintermedius is an opportunistic pathogen that is the major cause of pyoderma affecting dogs. Conventional antimicrobial treatment for infections caused by this organism have failed in recent years due to widespread resistance and alternative treatment strategies are a high priority. Protein A encoded in *Staphylococcus aureus* by *spa* protects the bacterium by binding IgG and acts as a superantigen. *S. pseudintermedius* possess two genes orthologous to *S. aureus spa, spsP,* and *spsQ.* The purpose of this study was to clone and express SpsQ and SpsQ-M, a non-toxigenic SpsQ, measure their cytotoxic effect on canine B cells and to evaluate antibody raised against them in clinically healthy dogs. *S. pseudintermedius* SpsQ induced apoptosis of canine B cells. Specific amino acid substitutions diminished SpsQ-M binding to immunoglobulin and its super-antigenic activity, while its antigenicity was maintained. This recombinant, non-toxigenic *S. pseudintermedius* SpsQ stimulated the production of antibodies in dogs that specifically reacted with SpsQ and greatly diminished its cytotoxic effect on canine B cells. These results suggest that attenuated SpsQ produced in this study is a good candidate for inclusion in a vaccine for use in the treatment and prevention of *S. pseudintermedius* infections.

Keywords: Protein A, SpsQ, *S. pseudintermedius*, B cells, immune evasion, virulence, vaccine

Introduction

S. pseudintermedius, a gram-positive, coagulase-positive bacterium, is commonly found on the normal skin and nasal flora of household pets (19, 79). It is the major cause of canine skin and ear infections (19, 80). Furthermore, human infections are sporadically reported (24, 81), suggesting that, although it is estimated to be low, there is a threat of zoonotic disease. Approximately 30-35% of the *S. pseudintermedius* isolates tested in our University of Tennessee College of Veterinary Medicine Clinical Bacteriology Laboratory are methicillin-resistant. Methicillin resistant *S. pseudintermedius* (MRSP) are resistant to most antimicrobial agents accessible at present and the number of multidrug resistant staphylococcal isolates is increasing (82-85).

Alternative approaches to antimicrobial therapy to treat staphylococcal infections, such as vaccines, have been difficult to develop. This is likely rooted in the ability of the bacteria to evade and/or destroy important components of their host defenses (86, 87). Protein A is anchored on the peptidoglycan cell wall and is also secreted during the exponential growth phase (88-91). It is an important virulence factor, able to bind IgG via its Fc region (21, 92, 93) protecting staphylococci from opsonophagocytosis. It also binds to and crosslinks the VH3-family of immunoglobulin idiotypes (the Ig fragment responsible for antigen recognition), triggering apoptosis (94, 95). Consequently, protein A plays an important role in enhancing pathogenicity of staphylococci by impairing the development of the host immune response (25, 86). Recently, it has been demonstrated that immunization with nontoxigenic *S. aureus* protein A (SpA_{KKAA}) (with amino acid substitutions in five repeated domains, E, D, A, B, and C) protected guinea pigs in experimental models of *S. aureus* infection (96). Also, *S. aureus* expressing the mutant variant was deficient in abscess formation, and laboratory animals infected with these mutant bacteria overcame the immunosuppressive effects of protein A and developed adaptive immune responses (96). Likewise, Pankey *et al*. (97) investigated the use of *S. aureus* protein A (SpA) as a vaccine for bovine mastitis with promising results.

In vertebrates, the immunoglobulin variable region of the heavy chain (VH) is encoded by diverse genes and the VH differ in length and amino acid composition between species and each species has it is own repertoire (98-100). In dogs, the VH1-family of immunoglobulin idiotypes represent the largest portion of VH genes in B cell populations (99).

Previous studies on SpA (101) have demonstrated that SpA binds to the Fab portion of VH3-type IgM on the surface of B cells resulting in cross-linking of B cell receptors that lead to B cell clonal expansion and subsequent apoptotic collapse, thereby limiting the ability of the host to produce antibody to other staphylococcal antigens (25).

In 2011, Bannoehr *et al.* (20) found that *S. pseudintermedius* possess two genes orthologous to *S. aureus spa*, *spsP* (encoding SpsP) and *spsQ* (encoding SpsQ, analogous to SpA). Balachandran *et al.* (21) recently found that SpsP and SpsQ consist of approximately 377 and 462 amino acids, respectively. Both proteins have predicted N-

terminal sequences of 33 amino acids, followed by a repeat region of three or four IgG-binding domains and a C terminal region that shares about 63% sequence similarity to the X-region of *S. aureus.*

The Immunoglobulin-binding domains (IgBDs) are highly conserved between SpsP and SpsQ, with amino acid sequence identity ranging from 67% to 90%. Balachandran *et al.* (21) found that *spsQ* was present in all clinical isolates of *S. pseudintermedius* tested within diverse multilocus sequence types, although they had different levels of expression, whereas *spsP* was less conserved and often occurs in degenerate form. They showed that *S. pseudintermedius* SpsQ is cell wall anchored and was detected in the bacterial secretome during log phase (21).

SpsQ bound to canine IgG primarily via its Fc region (21). This interaction was inhibited by anti-SpA raised in chickens which made *S. pseudintermedius* more susceptible to phagocytosis (21). Grandolfo (102) suggested that *S. pseudintermedius* SpsQ could be considered a possible vaccine target, however, information about SpsQ is scarce and it is necessary to determine if there are toxic effects of *S. pseudintermedius* protein A on canine B cells that can be neutralized with antibody.

The purpose of this study was to characterize the cytotoxic effect of recombinant *S. pseudintermedius* SpsQ on dog B cells, develop attenuated SpsQ (SpsQ-M), and evaluate antibody raised against SpsQ-M in clinically healthy dogs. *S. pseudintermedius* SpsQ-M was tested for its antigenicity and B cell killing. The results from this study suggest that *S. pseudintermedius* SpsQ may serve as a key component in a vaccine or as part of an immunotherapeutic treatment.

Results

Bioinformatics Analysis and *S. pseudintermedius* Protein A characteristics

S. pseudintermedius ED99, representing isolate that expresses both SpsQ and SpsP, SpsQ contained four and SpsP had genes encoding a maximum of three Ig-binding domains **(Figure 2.1, a)**. This compares to five domains with AA identities of 74% in *Staphylococcus aureus subsp. aureus* strain *Newman* SpA **(Figure 2.1, b)**. Three out of four SpsQ domains share 84-88% identity with the C domain of *S. aureus* SpA with

threonine at position 19, while only one domain is more similar to the B domain of *S. aureus* SpA **(Figure 2.1, c)**.

Multiple sequence alignment (MSA) between each domain of SpsQ and domain C of SpA, the most similar domain to SpsQ IgBDs, showed that each domain in SpsQ has binding sites for the IgG Fcγ (Glutamine (Q) $_{5}$ and Q $_{6}$), and Fab region (aspartate (D) $_{32}$ and D$_{33}$) of surface membrane-associated variable heavy 1 (VH1)-encoded B cell antigen receptors **(Figure 2.1, c)**. Furthermore, as with SpA of *S. aureus*, the secondary structure of SpsQ consists only of alpha helices and each Ig binding domain consists of three helices **(Figure 2.1, d)**.

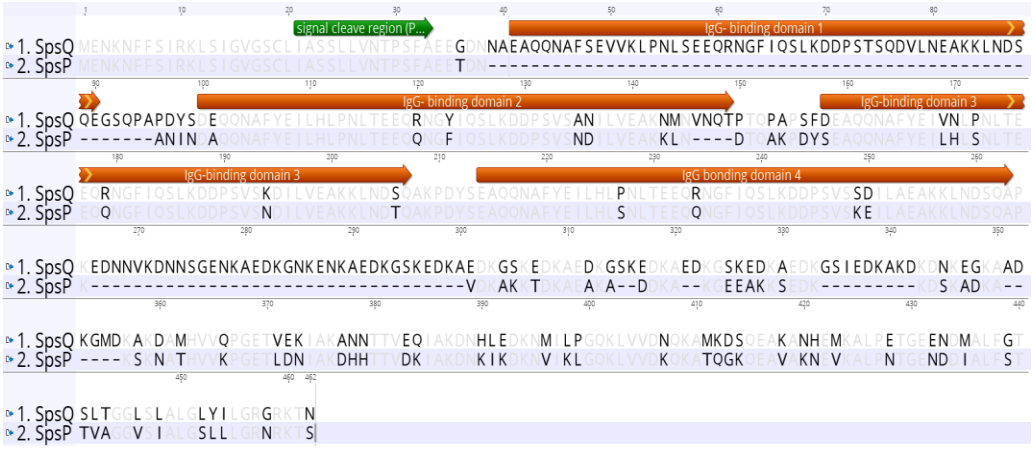


Figure 2.1 *S. pseudintermedius* SpsQ characteristics and basis for generation of recombinant nontoxigenic protein A (SpsQ)

(a) Pairwise alignment between *S. pseudintermedius* ED99 SpsQ and SpsP.

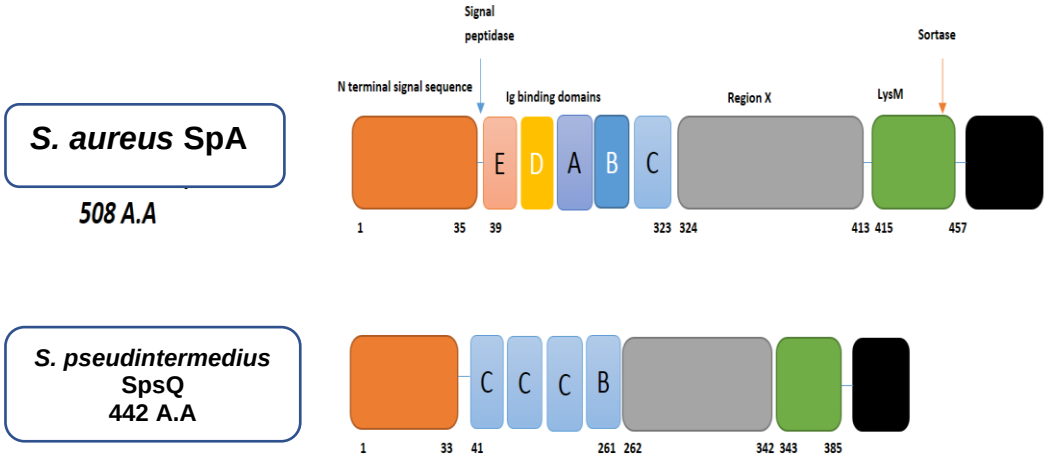


Figure 2.1 (b) SpA of *S. aureus* and SpsQ of *S. pseudintermedius* harbors an N-terminal signal peptide (orange boxes), Ig binding domains, variable region X (grey boxes), LysM sequence (green boxes) and C-terminal sorting signal (black boxes).

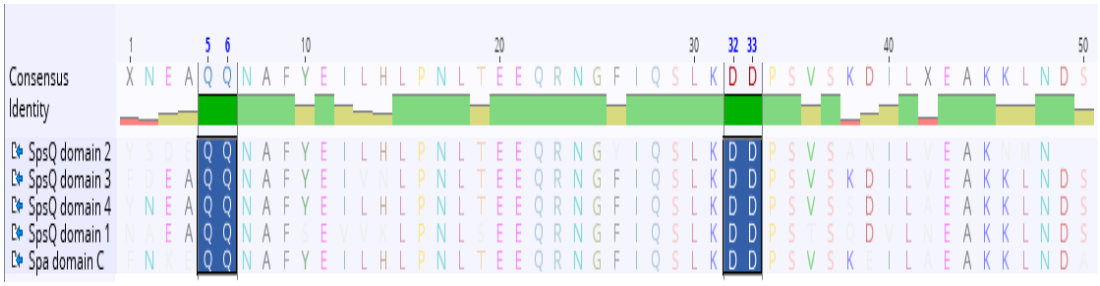


Figure 2.1 (c) Amino acid sequence of the four Ig binding domains, as well as Ig binding domain C of SpA, with the position glutamine (Q, highlighted in blue) 5 and 6 and aspartate (D, blue highlight) 32 and 33 as indicated.

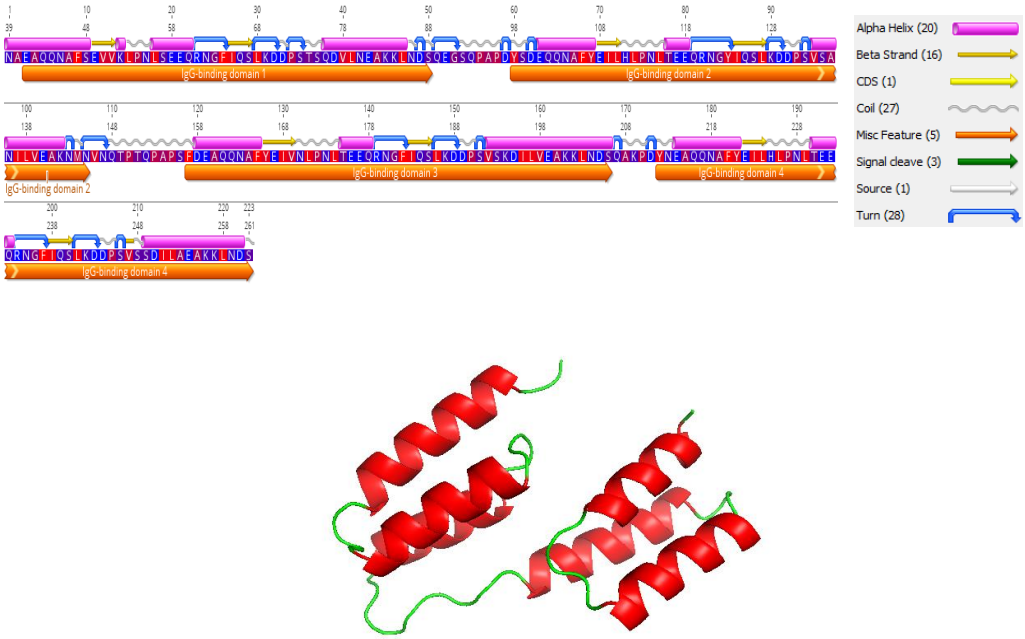


Figure 2.1 (d) SpsQ Secondary structure composition

Cloning, expression, and purification of recombinant SpsQ and SpsQ-M

Recombinant polyhistidine tagged SpsQ and SpsQ-M were produced in *E. coli* and purified using HisPur Ni-NTA resin under native conditions and eluted using an imidazole gradient. The molecular weights of SpsQ and SpsQ-M determined in western blots were of the expected sizes (47.2 and 47.6 kDa, respectively) **(Figure 2.2)**.

Antigenicity of SpsQ and SpsQ-M and their reactivity with serum from a dog with pyoderma

Chicken anti-protein A, chosen because protein A does not bind chicken immunoglobulin, had strong reactivity with affinity-purified SpsQ-M similar to that with *S. aureus* SpA and *S. pseudintermedius* SpsQ, indicating the attenuated protein retained its antigenicity **(Figure 2.3)**.

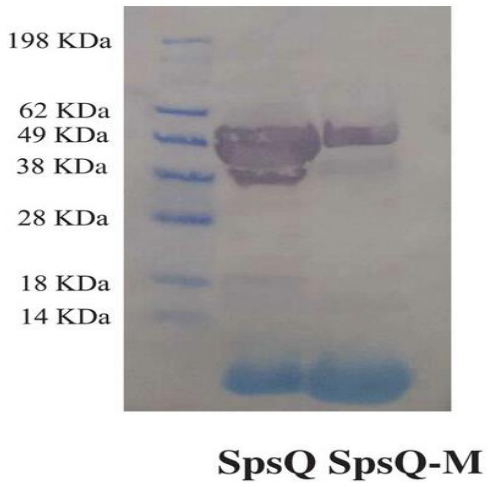


Figure 2.2 Western blot of recombinant *S. pseudintermedius* wild and mutant protein A with HRP-conjugated anti-6xhis tag monoclonal antibody, SpsQ and SpsQ-M were expressed in *E. coli* with M.W. of 47.2 and 47.6 KDa, respectively.

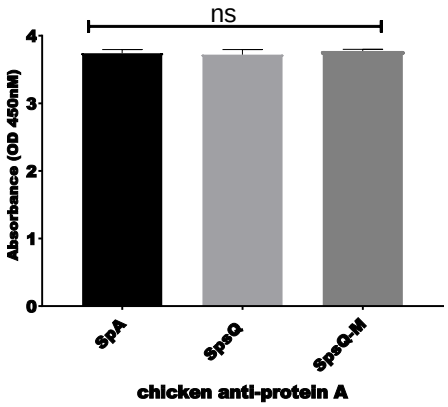


Figure 2.3 ELISA assay measuring the reactivity of HRP- conjugated chicken anti-protein A antibody with recombinant SpsQ, SpsQ-M and commercial *S. aureus* SpA coated on ELISA plates

The values represent average from three independent experiments. (*P < 0.05 was considered significant). ns – Not significant. There was no statistically significant difference between any sample P >0.05 (ns).

Sera from a dog with a history of chronic pyoderma showed significantly lower reactivity against SpsQ-M **(Figure 2.4 a)** compared to recombinant wild-type SpsQ and SpA. SpsQ-M had significantly lower binding (p-value of 0.0001) to commercial dog IgG **(Figure 2.4 a)**, IgM (p-value of 0.0001) **(Figure 2.4 b)** or IgA (p-value of 0.0001) compared to SpsQ and SpA. **(Figure 2.4 c)**.

S. pseudintermedius protein A induced apoptosis of canine B cells

Affinity-purified SpsQ-M had a low apoptotic effect on B cells at 1.5 h in contrast to recombinant SpsQ at the same time point, with a mean +/- SEM fluorescent intensity (MFI) difference of 2052 ± 285.2 between SpsQ and SpsQ-M (p-value of 0.0063) **(Figure 2.5, a)**. Incubation of the cells for 3.5 h with SpsQ-M resulted in a Sytox MFI reduction of 519,065 ± 128,292 SEM compared to that of SpsQ (p-value of 0.0003) **(Figure 2.5, b)**.

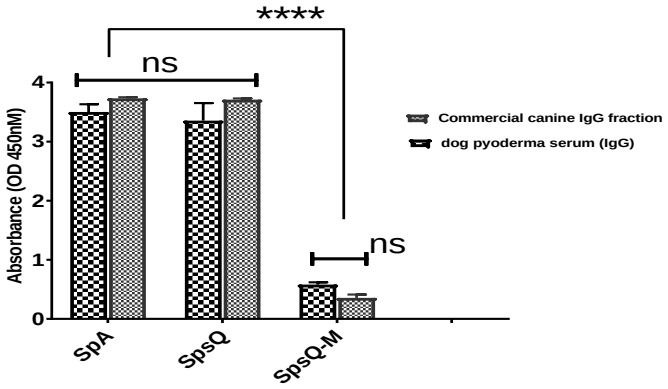


Figure 2.4 Infection with *S. pseudintermedius* did not elicit antibody specifically reactive with Staphylococcal protein A (a) *S. aureus* SpA and *S. pseudintermedius* recombinant SpsQ and SpsQ-M were coated on ELISA plates at 2 µg/ml in PBS. Then were incubated with serum from a dog with chronic pyoderma at a dilution of 1:2000, dog whole IgG molecule was used at the same dilution for comparison to measure non-specific IgG binding. Bound IgG were detected by HRP- Goat anti-dog IgG heavy and light chain. *S. aureus* SpA and *S. pseudintermedius* SpsQ reacted with both whole dog serum with chronic pyoderma and commercial canine IgG fraction with no significant difference (ns), however, attenuated SpsQ had significantly reduced nonspecific binding to both whole canine serum and canine IgG fraction (p value <0.0001) ****.

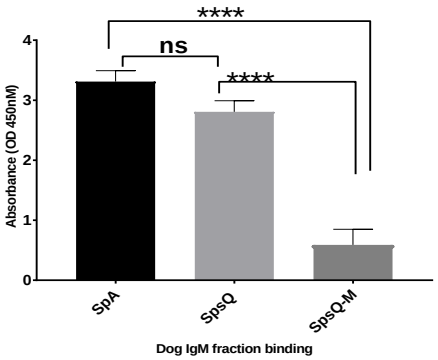


Figure 2.4 (b) *S. aureus* SpA and *S. pseudintermedius* SpsQ and SpsQ-M were coated on ELISA plates at 2 µg/ml in PBS. Then were incubated with serum from a dog with chronic pyoderma at a dilution of 1:2000. Bound IgM were detected by HRP-Goat anti-dog Goat anti-dog IgM µ chain.

Recombinant SpA and SpsQ bind to IgM from dog with pyoderma with no significant difference (ns) However, SpsQ-M significantly reduced nonspecific binding to IgM in dog serum (p value =0.0001) ****. The values represent average from three independent experiments. (*P < 0.05 was considered significant). ns – Not significant.

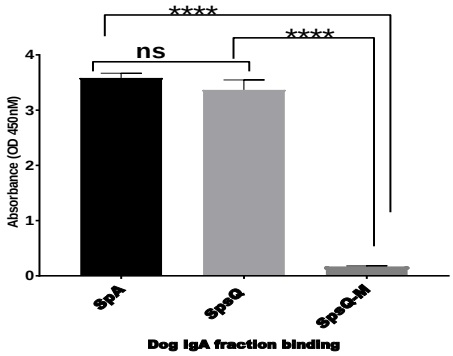


Figure 2.4 (c) SpA, SpsQ and SpsQ-M were coated on ELISA plates at 2 µg/ml in PBS. Then were incubated with serum from a dog with chronic pyoderma at a dilution of 1:2000. Bound IgA were detected by HRP-Goat anti-dog Goat anti-dog IgA. Recombinant SpA and SpsQ bind to IgA from dog with pyoderma with no significant difference (ns) However, SpsQ-M significantly reduced nonspecific binding to IgA in dog serum (p value <0.0001) ****. The values represent average from three independent experiments. (*P < 0.05 was considered significant). ns – Not significant.

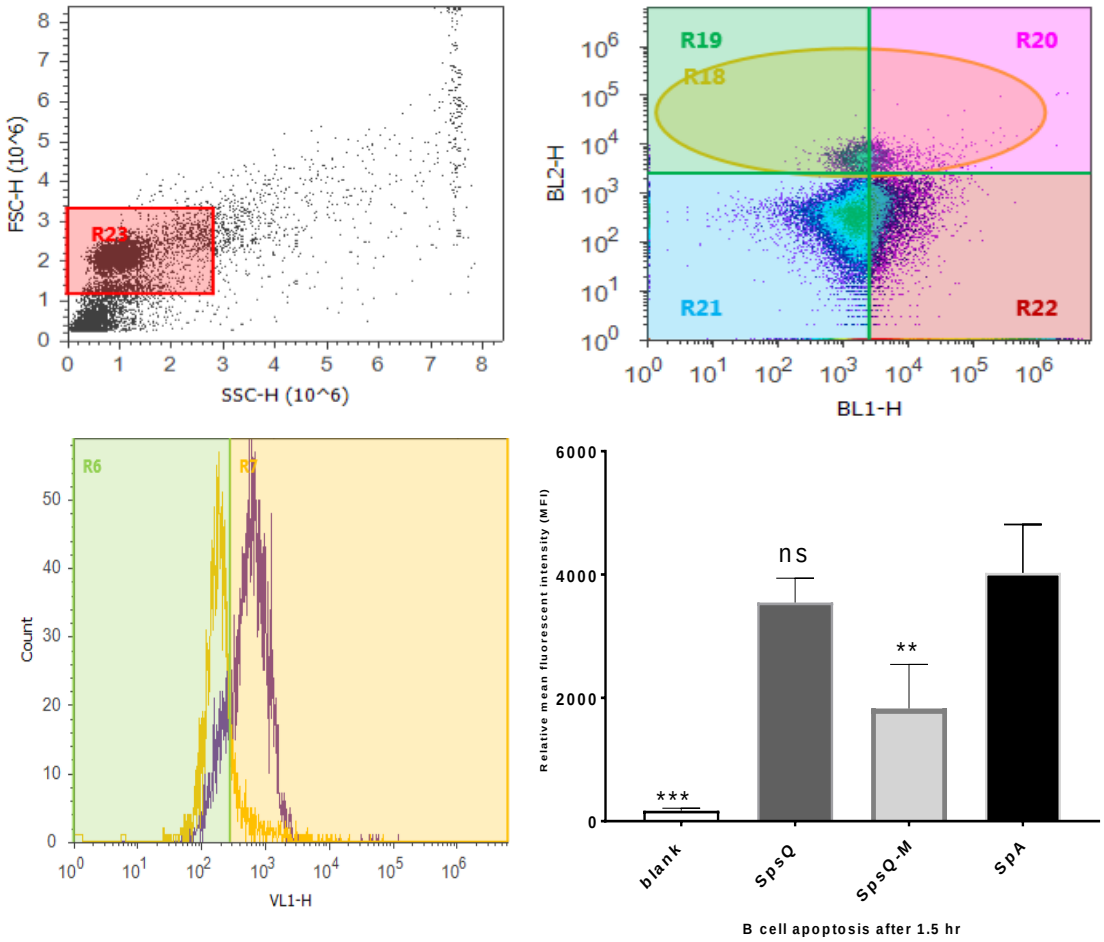


Figure 2.5 *S. pseudintermedius* recombinant SpsQ has a superantigenic effect on canine B cells (a) Gating on canine B cells using PE-anti-CD21 antibody, we found SpsQ induced B cells apopstosis after 1.5 hr (red peak on histogram) compared to SpsQ-M (violet peak on histogram). Mean Fluorescent Intensity (MFI) of blank, SpsQ and SpsQ-M relative to SpA was calculated based on average values from three independent experiments. (*P < 0.05 was considered significant). ns – Not significant. There was no statistically significant difference between SpA and SpsQ MFI after 1.5, P >0.05. However, MFI of SpsQ-M was significantly lower with P=0.0063 **.

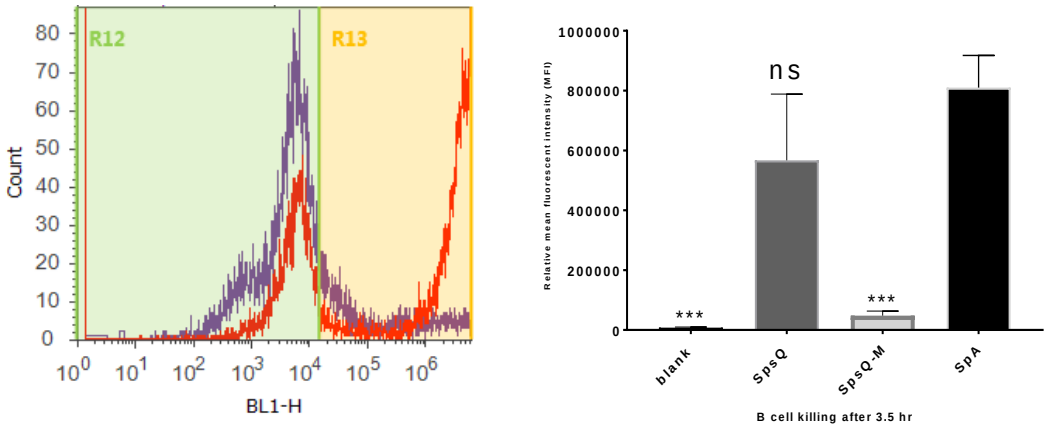


Figure 2.5 (b) Gating on canine B cells using PE-anti-CD21 antibody, we found SpsQ, with longer incubation for 3.5 hr, killed B cells (brown peak) compared to SpsQ-M (yellow peak) on histogram. MFI% of blank, SpsQ and SpsQ-M relative to SpA was calculated based on average values from three independent experiments. (*P < 0.05 was considered significant). ns – Not significant. There was no statistically significant difference between SpA and SpsQ MFI after 3.5 hr, P >0.05. However, MFI of SpsQ-M was significantly lower with P=0.0003***.

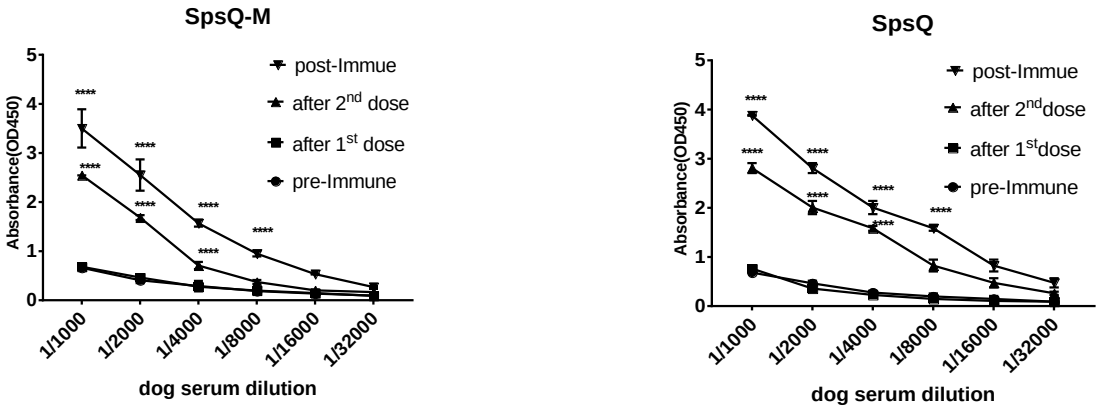
Specific antibody responses in dogs that reduce the effect of SpsQ on B cells *in vitro*

ELISA analysis of sera obtained from dogs on days -7, 8, 15 and 29 (relative to injections) showed that antibodies against *S. pseudintermedius* SpsQ-M and SpsQ were detected on day 15 and reached the highest level on day 29 (P = 0.0001) compared to pre-injection control sera **(Figure 2.6 a, b)**.

Pre-incubation of SpsQ, at a concentration of 100 µg/ml in PBS with dog anti-SpsQ-M resulted in 105665 ± 900.3 SEM, n=1 reduction in annexin mean fluorescent intensity (MFI) as compared with that of SpsQ treatment alone **(Figure 2.7)**.

Discussion

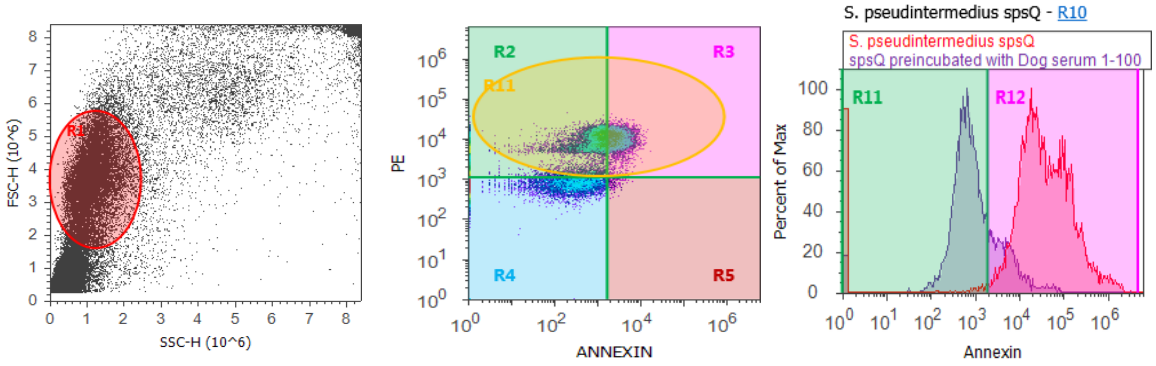
Protective host immune responses against extracellular pathogens are typically antibody-mediated. Unfortunately, staphylococcal infection does not usually establish protective immunity (98, 103-105) and efficacious staphylococcal vaccines have proven difficult to develop. Most vaccine research has focused on



a **b**

Figure 2.6 (a,b) Dog injected with SpsQ-M developed specific IgG reactive with native and mutant protein A

Specific antibodies against *S. pseudintermedius* SpsQ-M (**a**) and SpsQ (**b**) were detected using an indirect ELISA after the second injection (on day 15) and were higher on day 29 ($p = 0.0001$) compared to pre-injection control sera.



(a) (b) (c)

Figure 2.7. Dog anti- SpsQ-M antibody protects canine B cells from the superantigenic effects of SpsQ. Pre-incubation of SpsQ with dog anti-SpsQ-M resulted in mean 105,665 ± 900.3 SEM, n = 1 reduction in mean fluorescent intensity (MFI) from SpsQ on canine B cells compared to that of SpsQ treatment alone. **(a)** Gating on canine peripheral blood mononuclear cells (PBMC) based on side and forward scatter (shown in dot plot) and (**b)** on B cells using PE-anti-CD21 antibody (shown in density plot). **(c)** SpsQ induced B cell apopstosis after 1.5 hr (red peak on histogram) compared to SpsQ preincubated with dog serum at dilution 1:100 (blue peak on histogram).

S. aureus, which most commonly affects humans and is distinguished from *S. pseudintermedius,* which is primarily of veterinary concern.

Two forms of *S. pseudintermedius* protein A contain IgBDs that are similar to each other and likely to be antigenically cross-reactive. Because of their similarity and the presence of SpsQ in all tested isolates, SpsQ was used for this study. Understanding how *S. pseudintermedius* subverts the canine immune response is important for vaccine development and to facilitate natural host humoral immunity.

S. pseudintermedius SpsQ binding with the Fab region of surface membrane-associated VH1-encoded B-cell antigen receptors induce apoptosis in canine B cells. We developed a non-toxigenic SpsQ (SpsQ-M) by substitution of residues responsible for dual reactivity of each SpsQ domain with IgG Fcγ and Fab regions of surface membrane-associated VH1-encoded B-cell antigen receptors. Compared to staphylococcal protein A (106), SpsQ-M had much lower reactivity with IgG. Moreover, IgG, IgM, and IgA in sera from a dog with a history of chronic pyoderma had little immune-mediated binding to SpsQ-M indicating a lack of natural production of antibody elicited by infection with *S. pseudintermedius*. Comparing purified recombinant wild-type and mutant SpsQ, we found SpsQ-M had a significantly lower toxic effect on canine B lymphocytes. These results are in agreement with the findings of Kim *et al* (25) with *S. aureus* SpA (KKAA) as determined using mouse B lymphocytes and human immunoglobulins.

It should be noted that specific antibody directed against native protein A cannot be measured due to the non-immune binding of protein A to immunoglobulin. After injecting a clinically healthy dog with SpsQ-M, we observed a high titer of SpsQ-specific antibodies in agreement with Kim et al findings with SpAKKAA injected in mice (25). These results indicate that SpsQ, similar to SpA, suppresses adaptive immune responses during staphylococcal infection which may explain why previous *S. pseudintermedius* infections are not associated with protective immunity against recurrent infection.

Dog anti-SpsQ-M abolished the superantigenic effect of SpsQ that triggers apoptotic cell death in canine B cells, consistent with the findings of others with SpA studied in non-canine systems (25).

Collectively, the use of SpsQ-M will improve our understanding of the immune interactions between *S. pseudintermedius* and the dog. Data presented in our study suggest that blocking the Fcγ and Fab binding activities of SpsQ could be an alternative or adjunct to conventional antimicrobial treatments for infections caused by *S. pseudintermedius* including canine pyoderma (87). Canine anti-SpsQ-M may reduce SpsQ immune suppression in dogs, stimulate the production of specific antibodies against *S. pseudintermedius* and establish a protective immunity against recurrent infection. A SpsQ-M vaccine would likely contain additional *S. pseudintermedius* virulence factors.

Material and Methods

Ethics Statement. Experimental protocols were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC) including obtaining blood samples of dog (2474-0716) and injecting dogs with recombinant protein for producing antibodies (2572-1217).

Bioinformatics Analysis

MSA of *spsQ* from diverse isolates of *S. pseudintermedius* (n=100), was performed using Geneious, version 9.1.3(107). The bacterial localization prediction tool, PSORTb version 3.0.2 (http://www.psort.org/psortb/)(108), was used to determine the topology and domain structure of SpsQ and SpsP. SpsQ modeling and binding site prediction were performed using Protein Homology/analogY Recognition Engine V 2.0 (Phyre^2) (http://www.sbg.bio.ic.ac.uk/phyre2) (109), and the 3DLigandSite (http://www.sbg.bio.ic.ac.uk/3dligandsite/) (110), using SpA as a basis to predict the IgBDs in each domain. A pairwise sequence alignment of SpsQ and SpsP was used to identify conserved amino acids critical for IgG Fc and B cell receptor binding.

Identification of IgBDs was guided by *S. aureus* SpA secondary structure (111) and based on *S. aureus* SpA residues (25) responsible for dual reactivity of each domain in SpA with Fcγ (112) and Fab (113) and shared by *S. pseudintermedius* SpsQ.

Geneious, version 9.1.3 was used to select the locations for amino acids to be substituted in each IgBD and to design a full-length, four domains (SpsQ-M) attenuated *S. pseudintermedius* protein A construct (SpsQ-M). Glutamine (Q) 5 and 6, as well as aspartate (D) 32 and 33 in each domain of SpsQ were selected as critical amino acids for

the association of SpsQ with immunoglobulin. To test this, substitutions of Q5K (lysine), Q6K, D32A (alanine), and D33A were introduced into each IgBDs of SpsQ.

Bacterial strains and growth conditions

The *S. pseudintermedius* strain used in this study, strain 06-3228 (9), was isolated at the University of Tennessee, College of Veterinary Medicine Bacteriology Laboratory. It represents the most common multilocus sequence type (ST) previously reported in the United States (ST68) (82, 84, 114). Bacterial colonies grown on blood agar plates were inoculated into 5ml of sterile trypticase soy broth (TSB) (BD Biosciences, San Jose, CA, USA Cat No. RS1-011-21) and incubated overnight at 37°C with shaking at 225 rpm (Excella E24 Incubator Shaker, New Brunswick Scientific). Fifty microliters of overnight culture were inoculated into 5ml of fresh, sterile TSB to initiate log-phase bacterial cultures. Bacteria were grown at 37°C with shaking at 225rpm until an optical density of OD_{600} = 0.4-0.6 was reached (115).

Cloning, expression, and purification of recombinant wild-type and non-toxigenic *S. pseudintermedius* SpsQ

Bacteria from a single colony of *S. pseudintermedius* strain 06-3228 obtained from blood agar plates were grown in TSB at 37°C with 225 rpm shaking. DNA was extracted using a MO BIO UltraClean® Microbial DNA Isolation Kit (QIAGEN Inc., USA Cat No.12224-50) according to the instructions of the manufacturer. Oligonucleotide primers (Integrated DNA Technology, Coralville, USA) (**Table 2.1)** were designed using a PrimerQuest Tool (https://www.idtdna.com/Primerquest/Home/Index) based on the whole genomic sequence of *S. pseudintermedius* strain 06-3228 determined by Riley *et al*.(9).

The *spsQ* open reading frame (ORF) without the regions encoding the predicted N-terminal signal peptide was amplified from *S. pseudintermedius* 06-3228 genomic DNA. The ORF of *spsQ-M* was amplified from a PMA-*SpsQ-M* plasmid (**Table 2.2)** (Life Technologies Corp., Carlsbad, CA, USA), containing a synthetic *spsQ-M* gene. PCR was performed using taq polymerase (rTaq, Takara, USA Cat No. R004) and the following cycling conditions were performed: initial denaturation at 95°C for 90 seconds, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute followed by a final extension at 72°C for 5 minutes. All ORFs were

Table 2.1: Primers used to amplify recombinant wild and attenuated protein A (SpsQ) from *Staphylococcus pseudintermedius*.

Native full length *spsQ* forward	**GCATGAGGATCCAAGTTTCGCAGAAGAAGGAGATA**
Native full length *spsQ* reverse	**GCATGAGCGGCCGCACCGAATAATGCCATATCGTTT**
Attenuated full length *spsQ* forward	**GCATGAGGATCCAAGTTTCGCAGAAGAAGGAGATA**
Attenuated full length *spsQ* reverse	**GCATGAGCGGCCGCACCGAATAATGCCATATCGTTT**

Table 2.2 Plasmids and competent cells used to clone and express recombinant wild and attenuated protein A (SpsQ) from *Staphylococcus pseudintermedius*.

Plasmid/ Bacteria	**Expressed Gene**	**Source**
PMA-*spsQ-M*	Contain attenuated full length *S. pseudintermedius* protein A(SpsQ-M)	Synthetic gene, Life Technologies Corp., Carlsbad, CA
pETBlue-2	SpsQ and SpsQ-M expression with blue/white screening and C-terminal HSV•Tag® and His•Tag® sequences	Novagen, Madison, WI
Dh5-alpha Tuner™(DE3) pLacI	Cloning and recombinant SpsQ and SpsQ-M protein expression	Novagen, Madison, WI

amplified without a 6x histidine tag because pETBlue-2 (**Table 2.2)** allowed T7lac promoter-based expression of target genes with C-terminal histidine •Tag® sequences. PCR products were Sanger sequenced at The University of Tennessee Genomics Core facility.

To clone full length *S. pseudintermedius spsQ* and *spsQ*-M, the PCR products were digested with *NotI* and *BamHI,* then ligated into pETBlue-2, an expression vector with C-terminal HSV•Tag® and His•Tag® sequences (Novagen, USA Cat No .70674). The pETBlue-2 construct transformed into DH5-alpha *E. coli* chemically-competent cells (**Table 2.2)** (New England BioLabs Inc., USA Cat No .C2987I) by heat shock and DH5-alpha bacteria were plated on LB agar plates with 100 µg/mL ampicillin. The plasmid constructs were transformed into Tuner ™ (DE3) pLacI *E. coli* chemically-competent cells (**Table 2.2)** (Novagen, USA Cat No .70623) by heat shock and the bacteria were plated on LB agar containing 50µg/ml ampicillin and 20µg/ml chloramphenicol.

To express recombinant protein, a single colony of Tuner ™ (DE3) pLacI *E. coli* was inoculated into LB broth containing 50µg/ml ampicillin and 20µg/ml chloramphenicol and bacteria were grown overnight at 37ºC with 225 rpm shaking. LB broth containing 50µg/ml ampicillin and 20µg/ml chloramphenicol was inoculated with a 1:100 dilution of overnight culture and grown at 37ºC with 225 rpm shaking until a 600 nm optical density between 0.4 and 0.6 was reached. Protein expression was induced by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Teknova, USA Cat. No. I3431) and bacteria were grown for 4 h at 30ºC with shaking at 225 rpm. Bacterial cultures were centrifuged at 12,000 x g for 5 min in 5 ml of protein extraction reagent (BugBuster, Novagen , USA Cat No. 70584) and 20 µl of 100X protease inhibitor (Cocktail Set III, EDTA-Free Calbiochem, USA Cat No. 539134), and subsequently incubated for 30 min at 37ºC in a shaking incubator at 225 rpm. Bacteria were pelleted by centrifugation at 12,000 x g for 45 min at 4°C. Recombinant protein was purified from the supernatant using affinity purification (HisPur™ Ni-NTA Spin Purification Kit, Thermo Scientific, USA Cat No. 88228). Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Scientific, USA Cat No.23227).

Production of antibodies against recombinant proteins

Recombinant SpsQ-M at 100 µg/ 0.5 cc in phosphate buffered saline (PBS) (pH 7.2) was injected in the lateral thorax by the subcutaneous route, into three clinically normal dogs. Injections were given once every 7 days for a total of three injections with a control dog receiving PBS (pH 7.2) only. Blood (6 cc) was collected from a jugular vein using a 20 g needle and 12 cc syringe 4 times, on days -7, 8, 15 and 29. The collected blood was left undisturbed at room temperature for 30 min followed by centrifugation at 2,000 x g for 10 min in a refrigerated centrifuge.

SDS-PAGE and western blots

Protein samples were resolved by SDS-PAGE in 4-12 % polyacrylamide gels (Invitrogen, USA Cat No. NP0322BOX) and electrophoretically transferred onto nitrocellulose membranes (Thermo Scientific, USA Cat No. 77010). The blots were blocked overnight in 5% (w/v) nonfat dried milk powder dissolved in phosphate buffered saline containing 0.05% polyethylene glycol sorbitan monolaurate (Tween 20) (PBS-T) at 4°C. The blocked membranes were incubated with a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated anti-6xhis tag monoclonal antibody (Thermo Scientific, USA Cat No. MA1-21315-HRP) in 0.05% PBS-T for 1 h with 225 rpm shaking at room temperature. After five washes with 0.05% PBS-T, bound antibodies were detected using 1-Step™ chloronaphthol substrate solution (Thermo Scientific, USA Cat No. 34012).

Enzyme-linked immunosorbent assay

For measurement of the antigenicity of recombinant proteins, affinity-purified SpsQ and SpsQ-M were coated on ELISA plates (Corning, USA Cat No. 3590) at 2 µg/ml in PBS (pH 7.2). The plates were washed with PBS-T and incubated with HRP- chicken anti-protein A antibody (Gallus Immunotech, USA Cat No. APA) for 1 h at 37°C, then washed. For this and all subsequent ELISA assays, plates were washed three times with PBS-T between all incubations, bound antibodies were detected using TMB substrate (Thermo Scientific, USA Cat No. N301), reactions were stopped with 0.18 M sulphuric acid and optical density read at 450 nm on a plate reader (Bio TEK, USA Cat No. EL800). The experiment was repeated at least three times and a p-value of <0.05 was considered significant, which was the same for all the experiments unless mentioned otherwise.

To test the reactivity of serum from a dog with chronic pyoderma, *S. aureus* SpA and *S. pseudintermedius* SpsQ and SpsQ-M were coated on ELISA plates (Corning, USA Cat No. 3590) at 2 µg/ml in PBS. They were incubated with serum at a dilution of 1:2000. Bound IgG, IgM, and IgA were detected by HRP- goat anti-dog IgG-heavy and light chain (Bethyl Laboratories, Inc., USA Cat No. A40-123-1), HRP-goat anti-dog IgM µ chain (Bethyl Laboratories, Inc., USA Cat No. A40-116-2), HRP-goat anti-dog IgA (Bethyl Laboratories, Inc., USA Cat No. A40-104P) at a dilution of 1:8000 in PBS-T. Dog whole IgG molecule (Rockland, USA Cat No. 004-0102-1000) was used for comparison to measure non-specific IgG binding.

To detect a specific antibody response against SpsQ-M in injected dogs, recombinant *S. pseudintermedius* proteins and commercial SpA were coated on ELISA plates as previously described and incubated with two-fold serially diluted serum from injected dogs (1/1000-1/32000). Bound IgG was detected using HRP- goat anti-dog IgG-heavy and light chain (Bethyl Laboratories, Inc., USA Cat No. A40-123-1) with serum from uninjected dogs used as negative controls. The experiment was run in duplicate and a p-value of <0.05 was considered significant.

The ability of *S. pseudintermedius* SpsQ to kill B cells and induce B cell apoptosis

A total of 100 µg of purified recombinant SpsQ or SpsQ-M was mixed with isolated peripheral blood mononuclear cells (PBMC) in 1 ml of RPMI medium supplemented with 10% fetal bovine serum and incubated for 1.5 h at 37ºC in a 5% CO_2 incubator. To detect early phases of B cell apoptosis, phosphatidylserine was measured on the surface of cells using pacific blue- conjugated annexin (Thermo Scientific, USA Cat No. A35136). B cells were identified using phycoerythrin (PE) conjugated mouse anti-canine CD21 (clone: CA2.1D6) (BIO-RAD, USA Cat No. MCA1781PE) that recognizes canine CD21 (complement receptor type 2) on mature B lymphocytes. Stained B cells were analyzed using a flow cytometer (Attune acoustic focusing cytometer). B cells were also incubated with the same recombinant proteins as described above but for 3.5 h in order to detect B cell death. Cells were stained with Sytox green (Life Technologies, Inc., USA Cat No. 1776406) and PE-conjugated mouse anti-canine CD21. Gates were placed on cells positive for PE and these B cells were analyzed by flow cytometry.

To determine the protective effect of canine anti-SpsQ-M on B cells, recombinant *S. pseudintermedius* SpsQ was incubated for 30 minutes at 37ºC with serum from SpsQ-M injected dogs. The experiment was run in duplicate and a p-value of <0.05 was considered significant.

For flow cytometry analysis the cut-off for apoptosis or cell death was established using leukocytes incubated without SpsQ. Mean fluorescent intensity was determined from all B cells.

Statistical Analysis

A one-way ANOVA and Tukey–Kramer method were used to measure the significant differences between SpsQ, SpsQ-M, and SpA on inducing apoptosis and causing B cell death. However, two-way ANOVA and Tukey–Kramer methods were performed to test if there were significant differences in SpA, SpsQ or SpsQ-M binding with canine antibodies. All analyses were conducted using the GraphPad Prism software (Version 7, GraphPad Software Inc.).

Disclosure of interest

The authors report no conflict of interest.

Acknowledgments

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CHAPTER 3
CHARACTERIZATION OF A NEW LEUKOCIDIN IDENTIFIED IN *S. PSEUDINTERMEDIUS*

A version of this chapter was originally published by Mohamed A. Abouelkhair, David A. Bemis, Richard J. Giannone, Linda A. Frank, Stephen A. Kania:

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No revisions were made to the original published manuscript beyond general formatting to allow consistency through the dissertation. My contributions to this paper include: (i) selection of isolates (ii) experimental design and set up (iii) experimental testing (iv) data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) writing.

Abstract

Bacterial infections from *S. pseudintermedius* are the most common cause of skin infections (pyoderma) affecting dogs. Two component pore-forming leukocidins are a family of potent toxins secreted by staphylococci and consist of S (slow) and F (fast) components. They impair the innate immune system, the first line of defense against these pathogens. Seven different leukocidins have been characterized in *Staphylococcus aureus*, some of which are host and cell specific. Through genome sequencing and analysis of the *S. pseudintermedius* secretome using liquid chromatography mass spectrometry we identified two proteins, named “LukS-I” and “LukF-I”, encoded on a degenerate prophage contained in the genome of *S. pseudintermedius* isolates. Phylogenetic analysis of LukS-I components in comparison to the rest of the leukocidin family showed that LukS-I was most closely related to *S. intermedius* LukS-I and *S. aureus* LukE and LukP, whereas LukF-I was most similar to *S. intermedius* LukF-I and *S. aureus* gamma hemolysin subunit B. The killing effect of recombinant *S. pseudintermedius* LukS-I and LukF-I on canine polymorphonuclear leukocytes was determined using a flow cytometry cell permeability assay. The cytotoxic effect occurred only when the two recombinant proteins were combined. Engineered mutant versions of the two-component pore-forming leukocidins, produced through amino acids substitutions at selected points, were not cytotoxic. Anti-Luk-I produced in dogs against attenuated proteins reduced the cytotoxic effect of native *S. pseudintermedius* leukotoxin, which highlights the importance of Luk-I as a promising component in a vaccine against canine *S. pseudintermedius* infections.

Introduction

S. pseudintermedius is the primary cause of pyoderma (skin infection), the most common canine dermatologic disease, and is also associated with urinary tract infections, wound and surgical site infections, external ear otitis, abscess formation, mastitis and endocarditis (6, 82, 84). Approximately 30- 35% of the *S. pseudintermedius* isolates tested in our University of Tennessee College of Veterinary Medicine Bacteriology Laboratory from patients are methicillin-resistant (MRSP) and high levels of resistance

occurs in other regions of the United States (84). The vast majority of MRSP are multidrug resistant and there are increasing numbers of pandrug-resistant isolates (82-84).

Alternative approaches to control staphylococcal infections, such as vaccines, have been difficult to develop. This is likely rooted in the ability of the bacteria to neutralize and/or destroy important components of their host defenses. Some staphylococcal toxins impact the innate immune system, the first line of defense against this pathogen (116-118). Antibody-mediated toxin neutralization may contribute to a strategy for immunotherapeutic prevention of current and recurrent infections (116, 119).

Leukocidins are a family of potent toxins contributing to the pathogenicity of staphylococci (120). Leukocidins consist of two classes of proteins designated as S and F subunits (50, 121, 122) based on their chromatographic elution properties where S and F stand for slow and fast-eluting proteins, respectively (123, 124). The subunits are produced and secreted separately. The S-component recognizes a receptor on the host cell, conferring high-affinity binding to the cell surface after which the F component is recruited to form octameric beta-barrel pores that penetrate the cell lipid bilayer into the plasma membrane leading to ion influx and efflux, apoptosis and ultimately cell death (50, 121, 122).

A total of seven different bicomponent pore-forming toxins (BCPFTs) have been identified and characterized in *Staphylococcus aureus* including HlgAB, LukMF, HlgCB, LukAB/HG, LukED, Panton-Valentine leukocidins (LukSF-PV/PVL), and LukPQ, some of which are host and cell specific (116). The encoding genes are located chromosomally (*hlgAB* and *hlgCB* and *lukAB/HG),* prophage associated (*pvl*, *lukPQ* and *lukMF)* or reside on a pathogenicity island (*LukED)* (50, 116-118, 121, 122).

A bi-component toxin (LukS-I + LukF-I) from *Staphylococcus intermedius* was identified and characterized previously (125). Descloux et al (126) reported the presence of a leukocidin encoding gene (LukS-I) in genomes of 15 different *S. pseudintermedius* strains (including *S. pseudintermedius* type strain CCUG49543T) isolated from dogs without physical characterization of the protein.

In a previous study Karauzum et al. rationally designed mutants of *S. aureus* LukS-PV and LukF-PV subunits (54) . They tested mutant versions of LukS-PV with a series of amino acids substitutions and found that LukS-mut9, with T28F/K97A/S209A, was highly

immunogenic and non-cytotoxic when mixed with LukF-PV (54). Rabbit immunoglobulin raised against LukS-PV reduced the cytotoxic effect of canonical combinations (gamma hemolysin A and B subunits, gamma hemolysin C and B subunits and LukE and LukD), non-canonical pairs (gamma hemolysin B subunit and LukE, gamma hemolysin C subunit and LukD and gamma hemolysin A subunit and LukD) on polymorphonuclear leukocytes (PMNs) (127). LukS-mut9 vaccines significantly protected in a mouse model of *S. aureus* USA300 sepsis and this effect could also be achieved by passive transfer of rabbit anti-LukS-mut9 antisera (54).

The purpose of the present study was to analyze the secretome of *S. pseudintermedius* by mass spectrometry (MS) to determine the abundance of secreted Luk-I, characterize the cytotoxic effect of recombinant *S. pseudintermedius* Luk-I on canine PMNs and develop attenuated, nontoxic LukS-I and LukF-I. Attenuated LukS-I and LukF-I were tested for their antigenicity and PMN killing. Furthermore, antibody raised against *S. pseudintermedius* attenuated Luk-I in clinically healthy dogs was evaluated for its ability to neutralize wild-type LukS-I and LukF-I. The results from this study suggest that *S. pseudintermedius* LukS-I and LukF-I may serve as key components in a vaccine or as part of an immunotherapeutic approach.

Materials and Methods

Ethics Statement.

Experimental protocols were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC) including obtaining blood samples from dogs (2474-0716) and injecting dogs with recombinant protein for producing antibodies (2572-1217).

Bacterial strains, plasmids and growth conditions

The *S. pseudintermedius* strains used in this study, representing the most common multilocus sequence types (ST) previously reported in the United States (82, 84, 114), included 06-3228 (ST68), 08-1661 (ST71) and NA45 (ST84) (82, 84, 114). Strains 06-3228 and 08-1661 were isolated at the University of Tennessee, College of Veterinary

Medicine Bacteriology Laboratory. Strain NA45 was a gift of Faye Hartmann of the University of Wisconsin, School of Veterinary Medicine.

Plasmid construct pMA- attenuated *LukS-I* and pMA- attenuated *LukF-I*, each containing a mutated, synthetic *S. pseudintermedius* gene (designed as described below) with BamHI/NotI cloning sites, was obtained commercially (Life Technologies Corp., Carlsbad, CA).

Bacterial colonies grown on blood agar plates were inoculated into 5ml of sterile trypticase soy broth (TSB) (BD Biosciences, San Jose, CA Cat No. RS1-011-21) and incubated overnight at 37°C with shaking at 225 rpm (Excella E24 Incubator Shaker, New Brunswick Scientific). Fifty microliters of overnight culture were inoculated into 5ml of fresh, sterile TSB to initiate log-phase bacterial cultures. Bacteria were grown at 37°C with shaking at 225 rpm until an optical density of OD_{600} = 0.4-0.6 was reached.

LC-MS/MS Analysis of *S. pseudintermedius* supernatant

Log-phase bacterial cultures of 06-3228, 08-1661 and NA45 were centrifuged at 10,000 x g for 30 minutes at 4°C (Sorvall RC-5C Plus Super Speed Centrifuge) and the supernatant was collected and passed through a 0.45µm filter (Whatman, GE Healthcare Lifesciences, Pittsburgh, PA). The filtrate was concentrated using an Amicon® ^{Ultra}-4 centrifugal filter (EMD Millipore Corp., Billerica, MA) and stored at -20°C until further analysis. Samples interrogating *S. pseudintermedius* supernatant/ secretome were prepared for shotgun LC-MS/MS analysis as previously described (128).

Peptides were separated and analyzed with a 2-step MudPIT LC-MS/MS protocol (salt cuts of 50 and 500 mM ammonium acetate) over a 4-hr period then measured with a hybrid LTQ XL-Orbitrap (Thermo Scientific, Waltham, MA) mass spectrometer (MS) at Oak Ridge National Laboratories, Oak Ridge, Tennessee, USA(129). Peptide fragmentation spectra were searched against sample-specific proteome databases (strains 06-3228, 08-1661 and NA45). Matching peptides (FDR < 1%) were assigned to proteins (130) and resulting secretomes compared. The percent coverage of detected proteins was calculated by dividing the number of amino acids in all found peptides by the total number of amino acids in the entire protein sequence.

Bioinformatics Analysis

Multiple sequence alignment (MSA) of mature LukS-I and LukF-I subunits of *S. pseudintermedius* 06-3228 strain with corresponding units in other leukotoxins **(Table 3.1)** was performed and a rooted phylogenetic tree (UPGMA (unweighted pair group method with arithmetic mean)) of Luk-I was generated with Geneious version 11.0.3 (107) with *S. pseudintermedius* protein A serving as an outgroup.

In order to identify the unique residues in the S-component of *S. pseudintermedius* Luk-I that may shape the protein function and specificity, an alignment of amino acid sequences of the DR4 region in the rim domain (an important region for S-component receptor binding) of *S. aureus* LukE, HlgA, LukM and LukP, *S. pseudintermedius* and *S. intermedius* LukS-I was performed.

The bacterial localization prediction tool, PSORTb version 3.0.2 (http://www.psort.org/psortb/) (131) was used to determine the topology and domain structure of LukS-I and LukF-I.

S. pseudintermedius LukS-I and LukF-I modeling and binding site prediction was performed using Protein Homology/analogY Recognition Engine V 2.0 (Phyre^2) (http://www.sbg.bio.ic.ac.uk/phyre2) (109) and the 3DLigandSite (http://www.sbg.bio.ic.ac.uk/3dligandsite/) using *S. aureus* LukSF-PV, LukED and LukPQ as a basis to predict the critical amino acids for protein function. PHAST (http://phast.wishartlab.com/index.html) and PHASTER (132, 133) (http://phaster.ca/) were used for prophage detection in a total of 22 *S. pseudintermedius* isolates.

Polymerase chain reaction (PCR) amplification of *LukS-I and LukF-I*

Bacteria from a single colony of *S. pseudintermedius* strain 06-3228 obtained from blood agar plates were grown in TSB at 37°C with 225 rpm shaking. DNA was extracted using a MO BIO UltraClean® Microbial DNA Isolation Kit (QIAGEN Inc. Cat No.12224-50) according to the instructions of the manufacturer. Oligonucleotide primers (Integrated DNA Technology, Coralville, USA) (**Table 3.2)** were designed using a PrimerQuest Tool (https://www.idtdna.com/Primerquest/Home/Index) (15) based on the genomic sequence of *S. pseudintermedius* strain 06-3228 (20).

Table 3.1 Leukotoxin subunits used in the rooted phylogenetic tree

Protein name	Species	Accession Number	Amino acid Length
bi-component leucocidins LukPQ subunit Q	***Staphylococcus aureus***	**WP_086037611.1**	**326**
bi-component leucocidins LukPQ subunit P	***Staphylococcus aureus***	**WP_086037612.1**	**311**
bi-component leucocidins LukED subunit E	***Staphylococcus aureus***	**WP_000473596.1**	**311**
bi-component leucocidins LukED subunit D	***Staphylococcus aureus***	**WP_099821693 .1**	**327**
bi-component leucocidins LukMF subunit M	***Staphylococcus aureus***	**WP_000476437.1**	**308**
bi-component leucocidins LukMF subunit F	***Staphylococcus aureus***	**WP_000694885 .1**	**322**
bi-component leucocidins LukSF- PV subunit LukS-PV	***Staphylococcus aureus***	**WP_000239544 .1**	**312**
bi-component leucocidins LukSF- PV subunit LukF-PV	***Staphylococcus aureus***	**WP_024937002 .1**	**327**
bi-component leucocidins Hlg-AB subunit Hlg-A	***Staphylococcus aureus***	**WP_000594519.1**	**309**
bi-component leucocidins Hlg-AB subunit Hlg-B	***Staphylococcus aureus***	**WP_000783426 .1**	**325**
***S. pseudintermedius* LukS-I**	***Staphylococcus pseudintermedius***	**WP_014613568.1**	**310**
***S. pseudintermedius* LukF-I**	***Staphylococcus pseudintermedius***	**WP_014613567 .1**	**326**
***S. intermedius* LukS-I**	***Staphylococcus intermedius***	**WP_019169129.1**	**310**
***S.intermedius* LukF-I**	***Staphylococcus intermedius***	**WP_086427464.1**	**325**

Table 3.2 Primers used in this study to amplify recombinant wild-type and attenuated LukS-I and LukF-I from *S. pseudintermedius*

NotI, XhoI and BamHI restriction sites are underlined.

***LukS-I* forward**	GCATGAGGATCCGGTAAAAAATAAATTATTAGCCGCAACA
***LukS-I* reverse**	GCATGAGCGGCCGCATTATGCCCCTTTACTTTAATTTCGTG
attenuated *LukS-I* forward	GCATGAGGATCCAAGGCCACGTGTCTTGTC
attenuated *LukS-I* reverse	GCATGAGCGGCCGCCCCATGAGGCCAGTCTTG
***LukF-I* forward**	GCATGACTCGAGAAAAGAATGGCTAATCAAATTACACCTGTATCTG
***LukF-I* reverse**	GCATGAGGATCCTTAGTGATGGTGATGGTGATGTACTGTATGCTGATCCCAATCAA
attenuated *LukF-I* forward	GCATGAGGATCCGGATCCAATGAAAATAAGCAAAGTTATC
attenuated *LukF-I* reverse	GCATGAGCGGCCGCGCGGCCGCTGATGGGTTTTTT

The native *LukS-I* and *LukF-I* open reading frames (ORF) (933 and 981 bp, respectively) without the regions encoding the predicted N-terminal signal peptide were amplified from *S. pseudintermedius* 06-3228 genomic DNA and the ORF of mutant *LukS-I* and *LukF-I* were amplified from pMA-*attenuated LukS-I* and pMA-*attenuated LukF-I* plasmids (Life Technologies Corp., Carlsbad, CA), respectively (**Table 3.3**).

PCR was performed using taq polymerase (rTaq, Takara, Cat No. R004) and the following cycling conditions were performed: initial denaturation at 95°C for 90 seconds, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute followed by a final extension at 72°C for 5 minutes. All ORFs were amplified without a histidine tag because pETBlue-2 allowed T7lac promoter-based expression of target genes with C-terminal histidine •Tag® sequences. PCR products were Sanger sequenced at The University of Tennessee Genomics Core facility.

Table 3.3 Plasmids and competent cells used to clone and express recombinant wild-type and attenuated *S. pseudintermedius* LukS-I and LukF-I

Plasmid/ Bacteria	Expressed Gene	Source
pMA- attenuated *LukS-I*	Contain attenuated *S. pseudintermedius LukS-I*	Synthetic gene, Life Technologies Corp., Carlsbad, CA
pMA- attenuated *LukF-I*	Contain attenuated *S. pseudintermedius LukF-I*	Synthetic gene, Life Technologies Corp., Carlsbad, CA
pETBlue-2	Native and attenuated LukS-I and LukF-I expression with blue/white screening and C-terminal HSV•Tag® and His•Tag® sequences	Novagen, Madison, WI
DH5-alpha	Cloning and recombinant proteins (wild-type and attenuated LukS-I and native LukF-I) expression	Novagen, Madison, WI
Tuner™(DE3) pLacI		
pKLAC2	An integrative expression vector of *S. pseudintermedius* LukF-I in yeast	New England Biolabs, Ipswich, MA
Kluyveromyces lactis	An expression host of *S. pseudintermedius* LukF-I	New England Biolabs, Ipswich, MA

Cloning, expression and purification of recombinant native and attenuated *Luk-I*

To clone *S. pseudintermedius* native and mutant LukS-I and mutant LukF-I, their PCR products were digested with NotI and BamHI, then ligated into pETBlue-2 (Novagen, Cat No .70674) and transformed into DH5-alpha *E. coli* chemically-competent cells (**Table 3.3)** (New England BioLabs Inc., Cat No .C2987I) by heat shock. The DH5-alpha *E. coli* were plated on LB agar plates with 100 µg/mL ampicillin. The plasmid constructs were transformed into Tuner ™ (DE3) pLacI *E. coli* chemically-competent cells (**Table 3.3)** (Novagen, Cat No .70623) by heat shock and the Tuner ™ (DE3) pLacI *E. coli* were plated on LB agar containing 50µg/ml ampicillin and 20µg/ml chloramphenicol.

To express recombinant *S. pseudintermedius* native and mutant LukS-I and mutant LukF-I, a single colony of Tuner ™ (DE3) pLacI *E. coli* was inoculated into LB broth containing 50µg/ml ampicillin and 20µg/ml chloramphenicol and bacteria grown overnight at 37ºC with 225 rpm shaking. LB broth containing 50µg/ml ampicillin and 20µg/ml chloramphenicol was inoculated with a 1:100 dilution of overnight culture and grown at 37ºC with 225 rpm shaking until a 600 nm optical density of between 0.4 and 0.6 was reached. Protein expression was induced by addition of 1 mM Isopropyl β-D-1-thiogalatopyranoside (IPTG) (Teknova, Cat. No. I3431) and bacteria were grown for 4 hr at 30ºC with shaking at 225 rpm. Bacterial cultures were centrifuged at 12,000 x g for 5 min in 5 ml of protein extraction reagent (BugBuster, Novagen Cat No. 70584) and 20 µl of 100X protease inhibitor (Cocktail Set III, EDTA-Free Calbiochem, Cat No. 539134) and incubated for 30 min at 37ºC in a shaking incubator at 225 rpm. Bacteria were pelleted by centrifugation at 12,000 x g for 45 min at 4°C. Recombinant proteins were purified using affinity purification (HisPur™ Ni-NTA Spin Purification Kit, Thermo Scientific, Cat No. 88228).

Recombinant native LukF-I was cloned using an integrative expression vector (pKLAC2) and expressed in *Kluyveromyces lactis* (New England Biolabs, Cat No. E1000S). Recombinant protein was purified from *K. lactis* supernatant using affinity purification (HisPur™ Ni-NTA Spin Purification Kit, Thermo Scientific, Cat No. 88228). Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Scientific, Cat No.23227).

SDS-PAGE and western blot

Protein samples were resolved by SDS-PAGE in 4-12 % polyacrylamide gels (Invitrogen, Cat No. NP0322BOX) and electrophoretically transferred onto nitrocellulose membranes (Thermo Scientific, Cat No. 77010). The blots were blocked overnight in 5% (wt/vol) nonfat dried milk powder in 0.05% polyethylene glycol sorbitan monolaurate (Tween 20) containing phosphate buffered saline (PBS-T) at 4°C. The blocked membranes were incubated with a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated anti-6xhis tag monoclonal antibody (Thermo Scientific, Cat No. MA1-21315-HRP) in 0.05% PBS-T for 1 h with 225 rpm shaking at room temperature. After five washes with 0.05% PBS-T bound antibodies were detected using 1-Step™ chloronaphthol substrate solution (Thermo Scientific, Cat No. 34012).

Preparation of canine anti-*S. pseudintermedius* Luk-I

Recombinant attenuated LukS-I and LukF-I produced in *E. coli* were purified using affinity chromatography (as above) and endotoxin concentrations were measured using a ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (Genscript, Cat. No. L00350). Recombinant attenuated LukS-I and LukF-I at 20 µg each / 0.5 cc in phosphate buffered saline (PBS) (pH 7.2) were injected in the lateral thorax by the subcutaneous route, into three clinically normal dogs. Injections were given once every 7 days for a total of three injections with a control dog receiving PBS (pH 7.2) only. Blood (6 cc) was collected from a jugular vein 4 times, on days -7, 8, 15 and 29. The collected blood was left undisturbed at room temperature for 30 min followed by centrifugation at 2,000 x g for 10 min in a refrigerated centrifuge.

Enzyme-linked immunosorbent assay

For measurement of recombinant protein antigenicity, attenuated LukS-I and LukF-I were coated separately onto ELISA plates (Corning, Cat No. 3590) at 2µg/ml in PBS (pH 7.2). The plates were washed with 0.05% PBS-T and incubated with two-fold serial diluted serum from dogs (injected with recombinant proteins) for 1 h at 37°C, then bound IgG was detected using HRP-conjugated goat anti-dog IgG heavy and light chain (Bethyl Laboratories, Inc. Cat No. A40-123-1). ELISA assays plates were washed three times with PBS-T between all incubations, bound antibodies were detected using TMB

substrate (Thermo Scientific, Cat No. N301), reactions were stopped with 0.18 M sulphuric acid and optical density read at 450 nm on a plate reader (Bio TEK, EL800). The experiment was repeated a minimum of three times and a p-value of <0.05 was considered significant for all the experiments unless otherwise stated.

PMN cell permeability assay

Canine blood was collected from healthy dogs using a sterile blood collection system with EDTA anti-coagulant (BD Vacutainer). Then, 600 µl of dog blood was added to 1 ml of red blood cell lysing buffer (Hybri-Max™, Sigma-Aldrich, Cat No. R7757-100ML) for 30 min at 37°C in 15 ml sterile plastic tube, centrifuged and re-suspended in 1ml RPMI medium supplemented with 10% fetal bovine serum. PMNs were incubated with recombinant proteins (LukS-I and LukF-I, LukS-I alone, LukF-I alone, attenuated LukS-I, attenuated LukF-I and 1:2 *S. pseudintermedius* 06-3228 supernatant) in a volume of 500 µl in RPMI medium supplemented with 10% fetal bovine serum in a 5% CO_2 incubator for 30 min. Supernatant of *S. pseudintermedius* 06-3228 was harvested at log phase to test the toxic effect of secreted Luk-I. PMNs were stained with 1 µl of Sytox Green (Life technologies, Inc. Cat No. 1776406) for 30 min, washed with PBS (pH 7.2) twice and analyzed using a flow cytometer (Attune acoustic focusing cytometer). In order to measure the protective effect of anti-Luk-I on canine PMNs, recombinant LukS-I and LukF-I were incubated with canine anti- Luk-I at a dilution of 1:100 for 30 min at 37°C, then tested with the cell permeability assay as previously described.

Biotin labeling of *S. pseudintermedius* wild type and attenuated Luk-I and single components

Purified recombinant *S. pseudintermedius* wild type and attenuated LukS-I and LukF-I at 500 µg/ml in PBS (pH 7.2) were incubated with 50µl of 10mM EZ-Link Sulfo-NHS-LC-Biotin reagent (equal to 20 fold molar excess of biotin) (Thermo Scientific, Cat No. 21327) for 30 min at room temperature. Excess biotin was removed using an Amicon Ultra-0.5 Centrifugal Filter Unit with a 30 kDa molecular weight cut-off (Milipore sigma, Cat No. UFC5030). The biotin-labeled proteins were stored at -20°C until further use.

To test the binding of wild type and attenuated LukS-I and LukF-I to canine PMNs, biotin labelled recombinant proteins were incubated with PMNs from a clinical healthy dog for

30 minutes at room temperature, the cells were washed, then PMNs were incubated with 1:500 dilution of avidin-FITC conjugate (Sigma-Aldrich, A2050) at room temperature for 30 minutes in the dark. Unbound conjugate was removed by washing and the amount of binding was determined using a flow cytometer (Attune acoustic focusing cytometer).

Statistical Analysis

Data was analyzed using ANOVA tests and followed by post hoc multiple comparisons with Tukey's adjust. Diagnostic analysis was performed on residuals for checking normality and equal variance assumptions. Rank data transformation was applied when assumptions were violated. Statistical significance was identified at the level of 0.05. Analyses were conducted in JMP pro 14 for Windows (SAS institute Inc., Cary, NC).

Results

LC MS/MS data analysis of *S. pseudintermedius* culture supernatant

Secreted proteins, were isolated and digested to peptides with trypsin and analyzed by LC-MS/MS. The high mass accuracy of intact peptides and their fragmentation profiles were searched against genome-derived amino acid sequences for each protein in the sequenced genome of each strain. Identified peptides were then mapped back to their respective proteins and proteins quantified by both sequence coverage as well as area-under-the-curve abundance. Using this semi-quantitative data, along with categorizing each protein by their predetermined cellular location (pSORT), we were able to identify both LukS-I and LukF-I proteins in supernatant at relatively high levels. They occurred in rank 5 (LukF-I) and rank 7 (LukS-I) out of 92 total extracellular proteins identified (PSORT: extracellular, membrane/cell wall-associated, location unknown), putting both in the top 10% of the extracellular dataset. Thus, MS identification confirmed the extracellular existence of these proteins in all virulent strains and that they are indeed expressed at relatively high-levels, thus serving as candidates for further analysis (presented here and on-going).

The percent coverage of LukS-I and LukF-I were calculated by dividing the number of amino acids in all found peptides by the total number of amino acids in the mature protein sequence (**Table 3.4).**

Table 3.4 LC MS/MS analysis of *S. pseudintermedius* culture supernatant

	Coverage
06-3228 strain	
LukS-I	10.0
LukF-I	39.3
08-1661 strain	
LukS-I	56.2
LukF-I	46.0
NA45 strain	
LukS-I	59.4
LukF-I	62.3

LukS-I and LukF-I were secreted by *S. pseudintermedius* 06-3228, 08-1661 and NA45. Secretome proteins were compared among the three isolates using their respective genomes as reference databases, the percent coverage was calculated by dividing the number of amino acids in all found peptides by the total number of amino acids in the entire protein sequence.

Characterization of *S. pseudintermedius* Luk-I

Multiple sequence alignment (MSA) analysis showed that Luk-I is conserved among *S. pseudintermedius* strains including 06-3221, 08-1661 and NA45 with amino acid identities over 99.4 % between strains.

A 14.9 kb incomplete prophage (similar to Φ Staphy_96_NC_007057) was identified in the genome of *S. pseudintermedius* 06-3228. A BLAST search of Φ Staphy_96_NC_007057 using complete genome sequences of *S. pseudintermedius* strains available in the GenBank database and others sequenced in our lab but not yet published, revealed that approximately 7 Kb of the phage are present in all of the *S. pseudintermedius* isolates examined (a total of 22 isolates). They also contain the coding DNA sequences (CDS) of ascorbate-specific PTS system EII A, B and C components,

probable L-ascorbate-6-phosphate lactonase UlaG (L-ascorbate utilization protein G), phosphoglycerate mutase family 2 and hypothetical protein.

Phylogenetic analysis of Luk-I in comparison to the entire leukocidin family showed that *S. pseudintermedius* LukS-I is closely related to *S. intermedius* LukS-I, *S. aureus* LukE and LukP. However, *S. pseudintermedius* LukF-I is closely related to *S. intermedius* LukF-I and *S. aureus* gamma hemolysin subunit B **(Figure 3.1).**

Multiple sequence alignment (MSA) analysis showed that Luk-I in *S. pseudintermedius* is a unique leukotoxin and that each functional component shares considerable similarity with other staphylococcal leukotoxin fractions (**Table 3.5).**

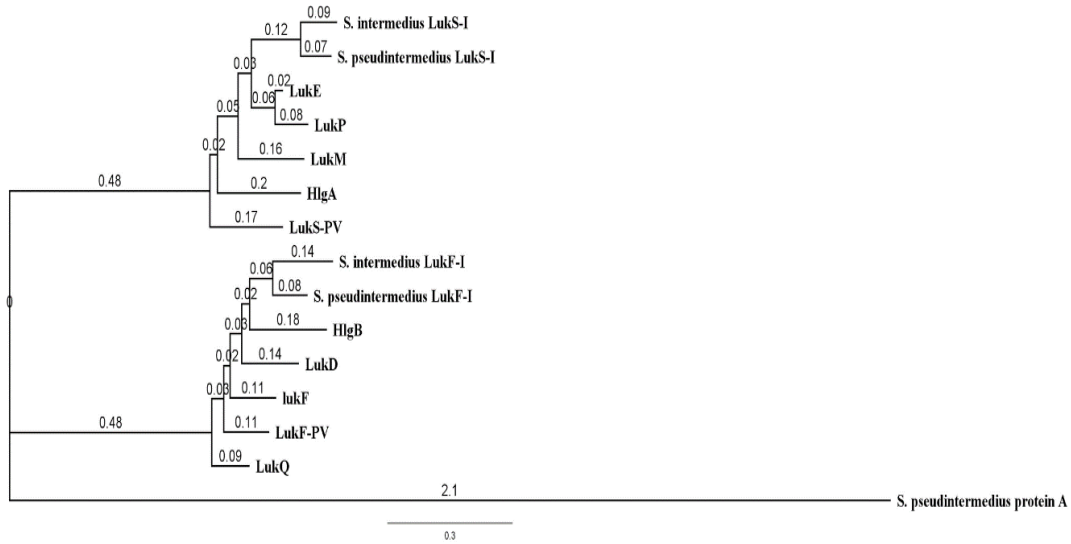

Figure 3.1 Phylogenetic tree based on amino acid sequences of mature leucocidins produced by *S. aureus*, *S. intermedius* and *S. pseudintermedius*.

S. pseudintermedius LukS-I is closely related to *S. intermedius* LukS-I, *S. aureus* LukE and LukP. *S. pseudintermedius* LukF-I is closely related to *S. intermedius* LukF-I and *S. aureus* gamma hemolysin subunit B. *S. pseudintermedius* protein A was used as an outgroup.

Table 3.5 MSA of LukS-I and LukF-I subunits of *S. pseudintermedius* 06-3228 strain with corresponding units in other leukotoxins

	LukP	hlgA	LukE	LukM	LukS-PV	*S. intermedius* LukS-I
***S.pseudintermedius* LukS-I**	**75.64%**	**73.08%**	**73.72%**	**68.49%**	**64.76%**	**85.48%**
	LukF-PV	**LukQ**	**LukF**	**LukD**	**hlgB**	***S. intermedius* LukF-I**
***S.pseudintermedius* LukF-I**	**73.60%**	**74.54%**	**73.46%**	**76.07%**	**72.62%**	**79.75%**

The protein sequence contains an N-terminal signal peptide sequence from positions 1 through 29 for LukS-I and 1 through 26 for LukF-I detected by SignalP 4.1 server (134). The LukF-I model developed with the Phyre^2 web portal, shows that LukF-I consists of β-strands organized as four antiparallel β-sheets and three very short α-helices **(Figure 3.2).** LukF-I, similar to the fold organization of other bicomponent pore-forming toxins, is arranged in three typical domains: β-sandwich (cap) (1-60, 79-107, 147-169, 220-246 and 569-300), stem (108-146) and rim (61-78, 170-219 and 247-268)) **(Figure 3.3, a).** The rim domain has critical residues for membrane binding, the β-sandwich (cap) domain plays a role in side by side residues interaction and oligomerization with S component, and the pre-stem domain is important for pore formation but not membrane-binding.

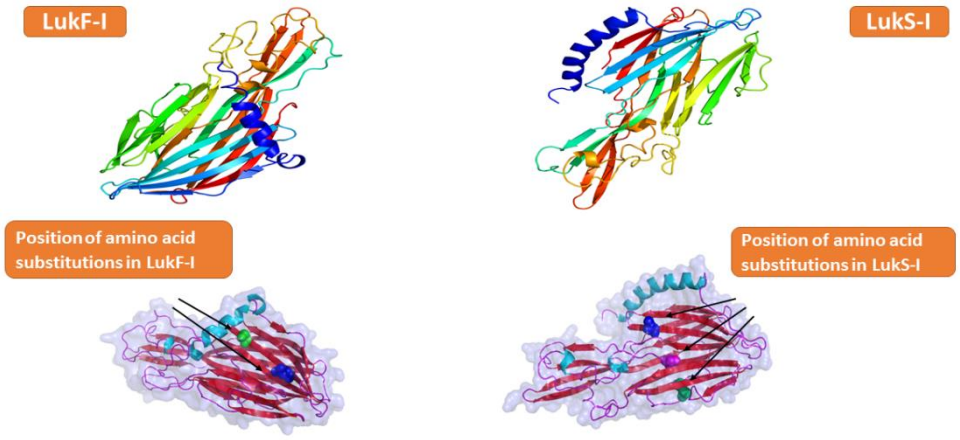

Figure 3.2 Ribbon representation of Wild-type and attenuated *S. pseudintermedius* LukS-I and LukF-I proteins.

Colour-ramped from the N terminus (blue) to the C terminus (red). The output structure was generated with Pymol.

In *S. pseudintermedius* LukF-I, we identified tyrosine (Y) 71, tryptophan (W) 176, arginine (R) 179, W256, phenylalanine (F) 259 and histidine (H) 260 as critical residues located in the rim domain, important for phospholipid binding on the membranes of canine PMNs while serine (S) 33 and asparagine (N) 39 located in the β-sandwich (cap) domain are essential for interaction with side residues of LukS-I which stimulate oligomerization. These residues are conserved among *S. intermedius* LukF-I and *S. aureus* LukD **(Figure 3.3, b).**

MSA of amino acid sequences of the divergent region 4 (DR4) in the rim domain (an important region for S-component receptor binding) of *S. aureus* LukE, HlgA, LukM and LukP, *S. pseudintermedius* LukS-I and *S. intermedius* LukS-I revealed that the DR4 regions of *S. aureus* LukP and LukS-I of *S. pseudintermedius* and *S.intermedius* are almost identical. By comparison, LukM, HlgA and LukE are considerably different **(Figure 3.3, c).**

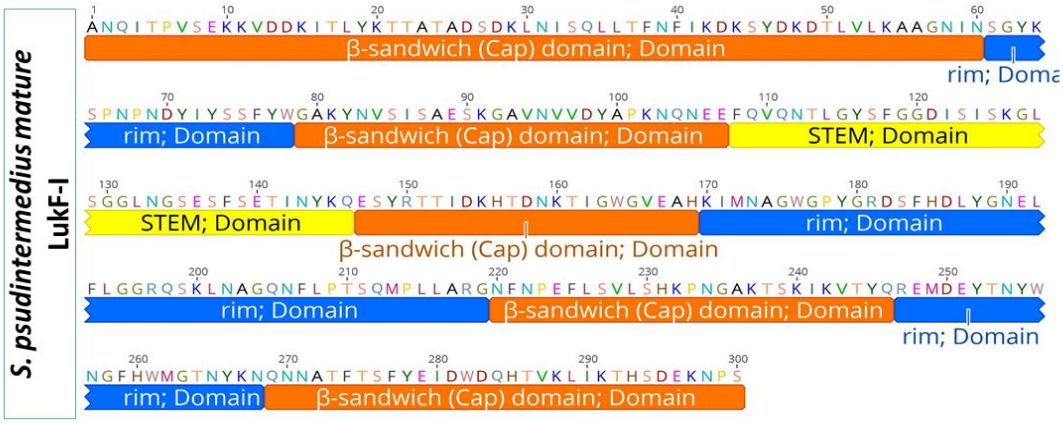

Figure 3.3 Unique residues in S and F-components of *S. pseudintermedius* Luk-I may shape the protein function and specificity

(a) domain structure of *S. pseudintermedius* LukF-I consist of β-sandwich (cap) domain highlighted in orange color (1-60, 79-107, 147-169, 220-246 and 569-300), stem highlighted in yellow (108-146) and rim highlighted in blue (61-78, 170-219 and 247-268).

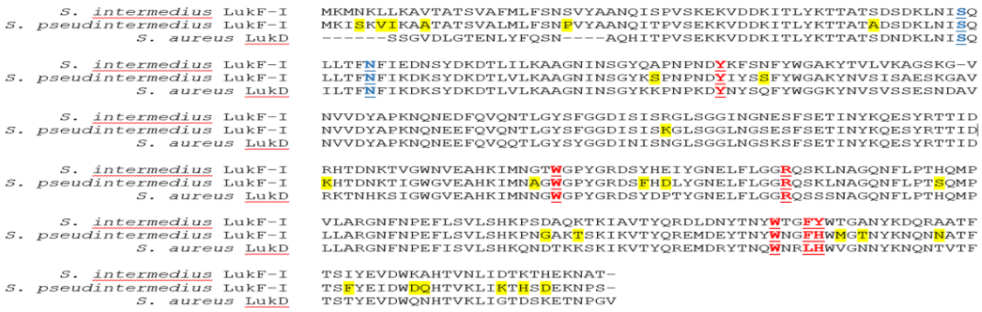

Figure 3.3 (b), Multiple sequence alignment (MSA) of *S. intermedius* and *S. pseudintermedius* LukF-I and *S. aureus* LukD. Geneious version 11.0.3 was used to generate the alignment. Residues unique to *S. pseudintermedius* LukF-I are highlighted yellow, residues identified as important for phospholipid interaction are shown in red and underlined text and residues important for oligomerization is highlighted in blue.



Figure 3.3 (c) MSA of amino acid sequences of the DR4 region (highlighted in yellow) in the rim domain (an important region for S-component receptor binding) of *S. aureus* LukE, HlgA, LukM and LukP, *S. pseudintermedius* LukS-I and *S. intermedius* LukS-I. The DR4 regions of *S. aureus* LukP and LukS-I of *S. pseudintermedius* and *S.intermedius* are almost identical, whilst that of LukM, HlgA and LukE are considerably different.

Design of LukS-I and LukF-I with amino acid substitutions

We designed attenuated LukS-I and LukF-I with mutations away from the rim region to disrupt the ability of LukS-I and LukF-I to interact side by side and form oligomers, a function critical for cytolysis.

S. pseudintermedius LukS-I and *S. aureus* LukS-PV share the critical residues T28, K99 and S211 for interaction and oligomerization with the corresponding F-component (54). Guided by the LuKS-I protein model developed by Phyre[2], and MSA of amino acid sequences of *S. pseudintermedius* LukS-I and *S. aureus* LukS-PV **(Figure 3.3, d)**, an attenuated *S. pseudintermedius* LuS-I was designed with the following substitutions: T28F, K99A and S211A.

Moreover, we identified serine (S) 59 and asparagine (N) 65 in the mature LukF-I protein as residues essential for side-by-side interactions with LukS-I to form oligomers. To test this, we designed attenuated LukF-I with the following substitutions: S59D and N65A.

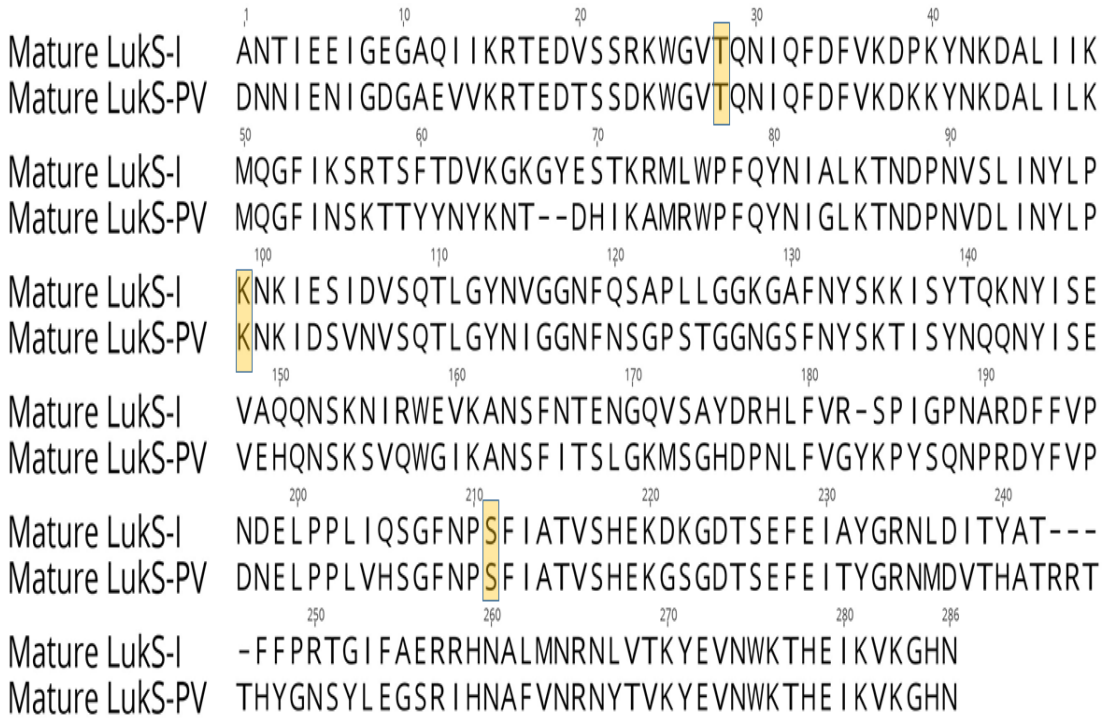

Figure 3.3 (d) MSA of amino acid sequences of *S. pseudintermedius* LukS-I and *S. aureus* LukS-PV showing the critical residues T28, K99 and S211 (highlighted in yellow) that interact with the corresponding F-component.

Cloning, expression and purification of recombinant *S. pseudintermedius* LukS-I and LukF-I

Recombinant polyhistidine tagged wild type and mutant LukS-I and LukF-I were generated in *E. coli* and LukF-I was secreted in the culture supernatant of *K. lactis* using an integrative expression vector (pKLAC2). Recombinant proteins were purified using HisPur Ni-NTA resin under native conditions and eluted using an imidazole gradient. The molecular weights of LukS-I, attenuated LukS-I, LukF-I and attenuated LukF-I, determined in western blots, were of the expected sizes (39.43, 39.12, 37.27 and 37.59 kDa, respectively) **(Figure 3.4)**. The endotoxin levels of purified recombinant, attenuated LukS-I and LukF-I were below 0.5 endotoxin units /mg protein.

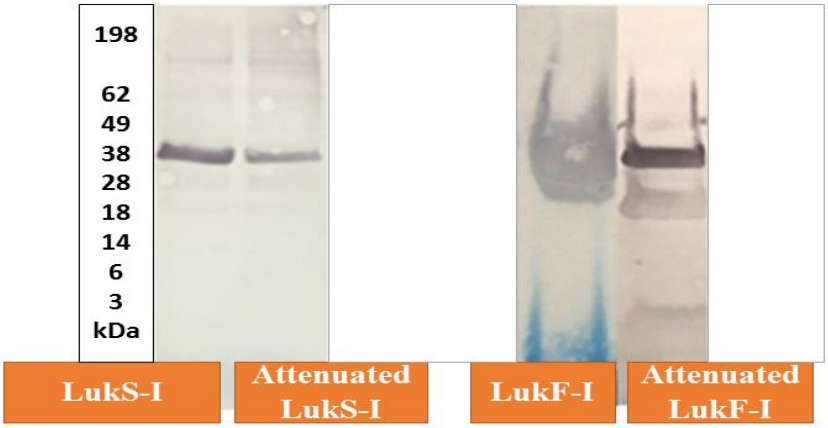

Figure 3.4 Western blot of recombinant *S. pseudintermedius* wild-type and attenuated LukS-I and LukF-I with HRP-conjugated anti-6xhis tag monoclonal antibody. The Molecular weights of LukS-I, LukF-I, attenuated LukS-I and attenuated LukF-I determined in western blots using SeeBlue® Pre-Stained Protein Standard (x 1,000) were of the expected sizes 39.43, 39.12, 37.27 and 37.59 kDa, respectively.

Attenuated Luk-I induces specific antibody responses

Specific antibodies against *S. pseudintermedius* wild type and attenuated LukS-I and LukF-I were measured by ELISA (described below) in sera collected on days -7, 8, 15 and 29, relative to antigen injections. Antibodies were detected after the second injection of attenuated Luk-I (on day 15) and were highest on day 29 ($P < 0.0001$) compared to pre-injection control sera **(Figure 3.5)**.

Luk-I kills canine PMNs

Canine PMNs were highly susceptible to Luk-I with lysis induced within 30 minutes at a concentration of 200 ng of each leukototxin component and a 1:2 *S. pseudintermedius* 06-3228 supernatant dilution **(Figure 3.6, a)**. The mean fluorescent intensity (MFI) of PMNs treated with wild-type LukS-I ($P = 0.0330$***) and LukF-I ($P = 0.0468$**) was significantly lower than that with Luk-I treatment. PMNs killing represented in MFI was significantly lower in attenuated LukS-I ($P = 0.0276$**), attenuated LukF-I ($P = 0.0268$**) and attenuated Luk-I treatments ($P = 0.0044$***) compared to that with 1:2 *S. pseudintermedius* 06-3228 supernatant dilution **(Figure 3.6, b)**.

Amino acid substitutions in *S. pseudintermedius* LukS-I and LukF-I did not abolish leukotoxin binding to canine PMNs

To test the effect of critical amino acid substitutions on attenuated LukS-I and LukF-I binding to canine PMNs, biotin labelled recombinant wild type and attenuated LukS-I and LukF-I were incubated with canine PMNs and their binding detected using FITC conjugated avidin. No significant difference in MFI of attenuated or wild type recombinant LukS-I and LukF-I was found ($P > 0.05$) **(Figure 3.7)**.

Dog Anti-Luk-I reduced the cytotoxic effect of canine leukotoxin on PMNs

Dog anti-Luk-I at a dilution of 1:100 preincubated with Luk-I showed a significant reduction in mean fluorescent intensity (MFI) compared with Luk-I treatment alone ($P = 0.0036$**) and with that of 06-3228 supernatant treatment alone ($P = 0.0002$***) **(Figure 3.8)**.

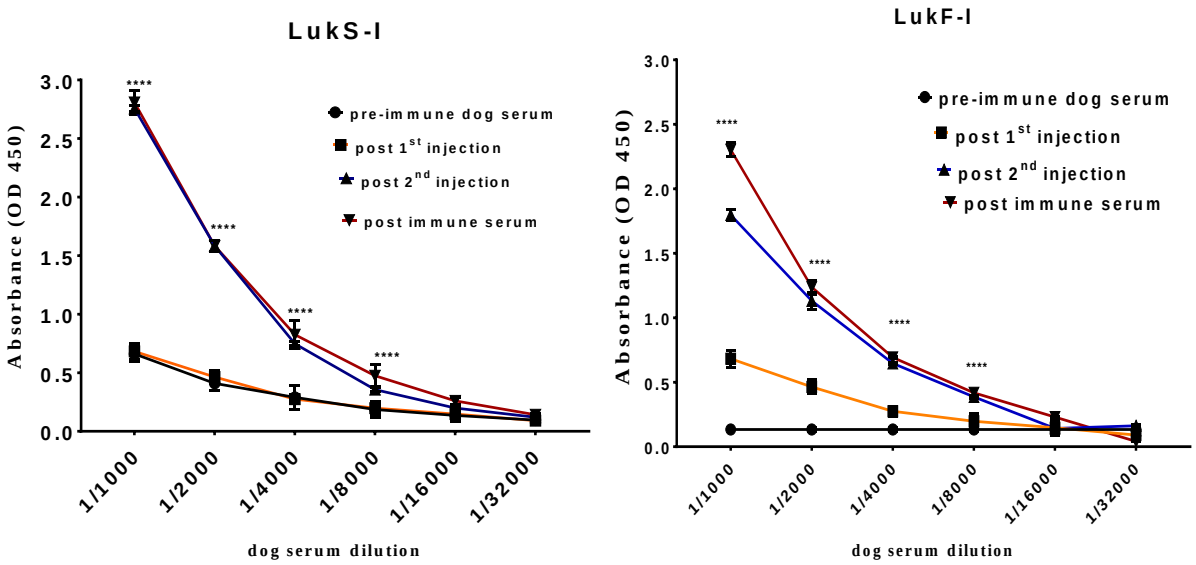

Figure 3.5 Canine antibody against attenuated LukS-I and LukF-I react with recombinant wild-type LukS-I and LukF-I.

Antibodies against *S. pseudintermedius* wild-type LukS-I and LukF-I were detected using an indirect ELISA. Recombinant *S. pseudintermedius* LukS-I and LukF-I proteins were coated on ELISA plates, then incubated with two-fold serially diluted serum from dog vaccinated with the same proteins. High reactivity with LukS-I and LukF-I was seen from sera collected two weeks after 3rd injections of attenuated LukS-I (P =0.0001****) and LukF-I (P =0.0001****) compared to pre-injection sera. The values represent averages from three independent experiments.

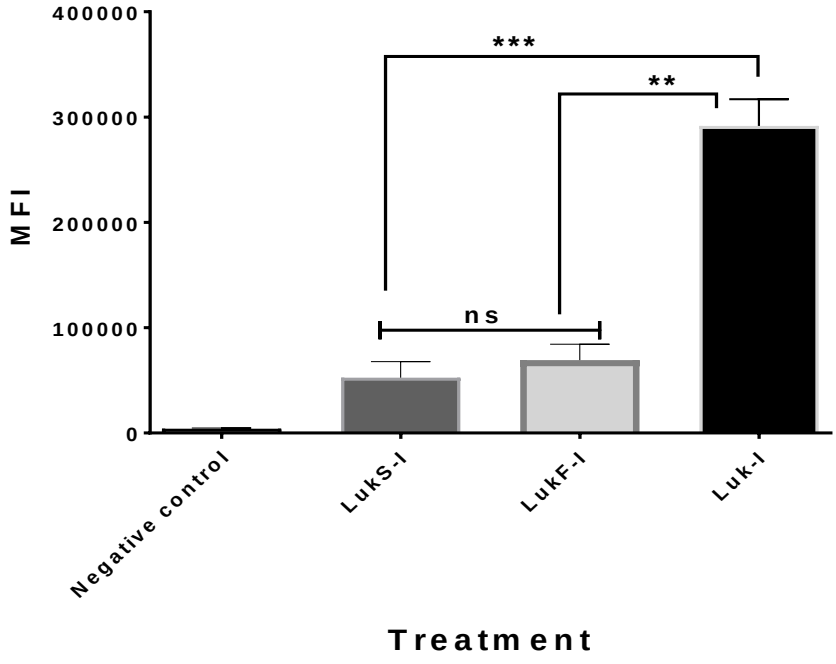

Figure 3.6 Cytotoxic effect of *S. pseudintermedius* recombinant Luk-I on canine PMNs. PMN permeability assay using Sytox Green to detect the cytotoxic effect of wild-type Luk-I on canine PMNs. (a) Luk-I significantly induced PMN killing after 30 min compared to that with wild-type LukS-I (P = 0.0330***) and LukF-I (P = 0.0468**).

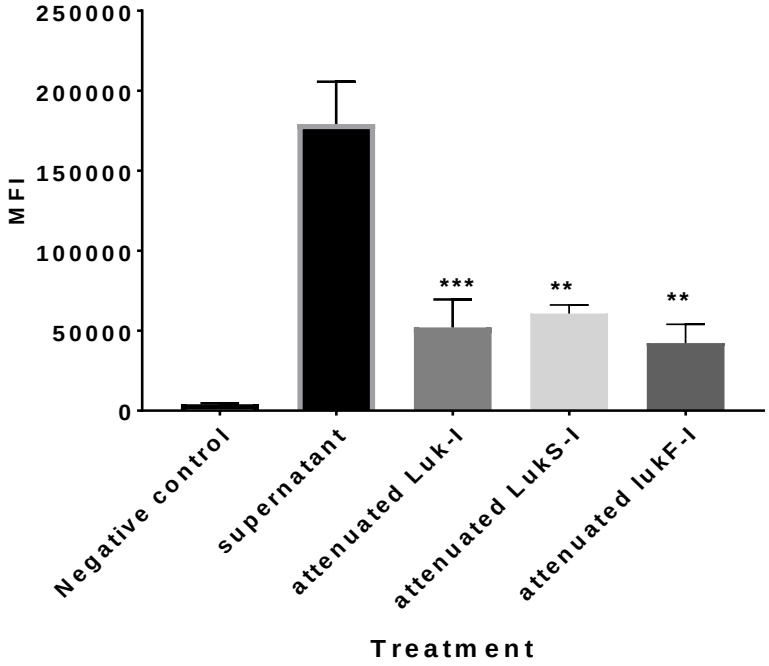

Figure 3.6 (b) A1:2 *S. pseudintermedius* 06-3228 supernatant dilution significantly induced PMN killing after 30 min compared to that with and attenuated LukS-I (P = 0.0276**), attenuated LukF-I (P = 0.0268**) and attenuated Luk-I treatments (P = 0.0044***)

The mean fluorescent intensity (MFI) of all treatment were calculated based on average values from three independent experiments. (*P < 0.05 was considered significant). ns – Not significant.

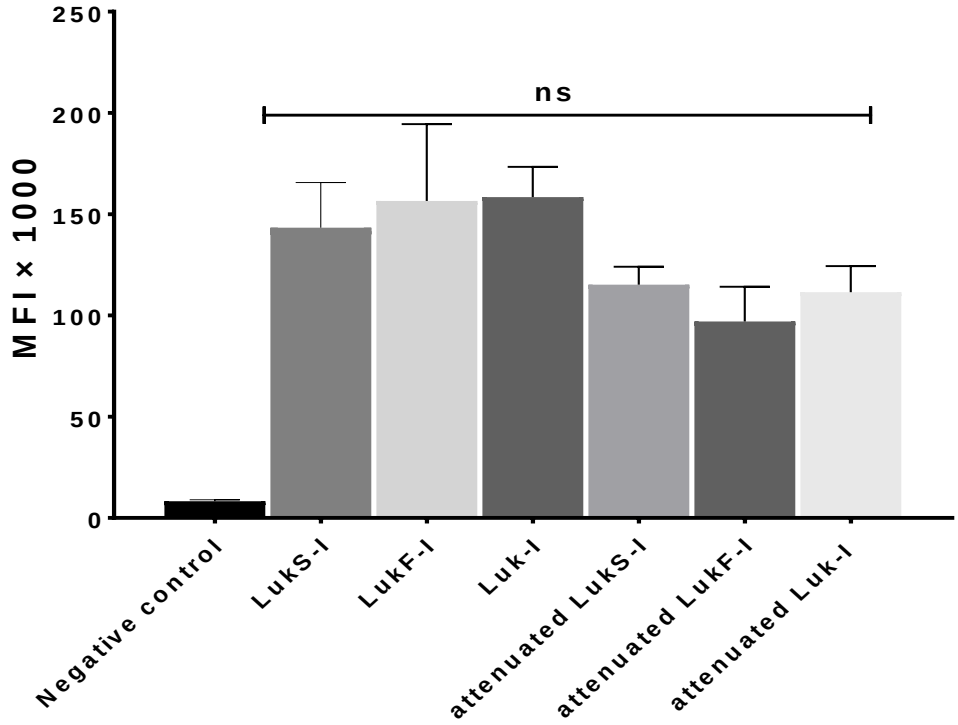

Figure 3.7 *S. pseudintermedius* wild-type and attenuated Luk-I binding to canine PMNs

Biotin labelled recombinant wild type and attenuated LukS-I and LukF-I were incubated with canine PMNs and their binding was detected using FITC conjugated avidin. MFI of the blank, wild-type and attenuated proteins were calculated based on average values from three independent experiments. All of the recombinant proteins bind to canine PMNs with no significant difference (ns).

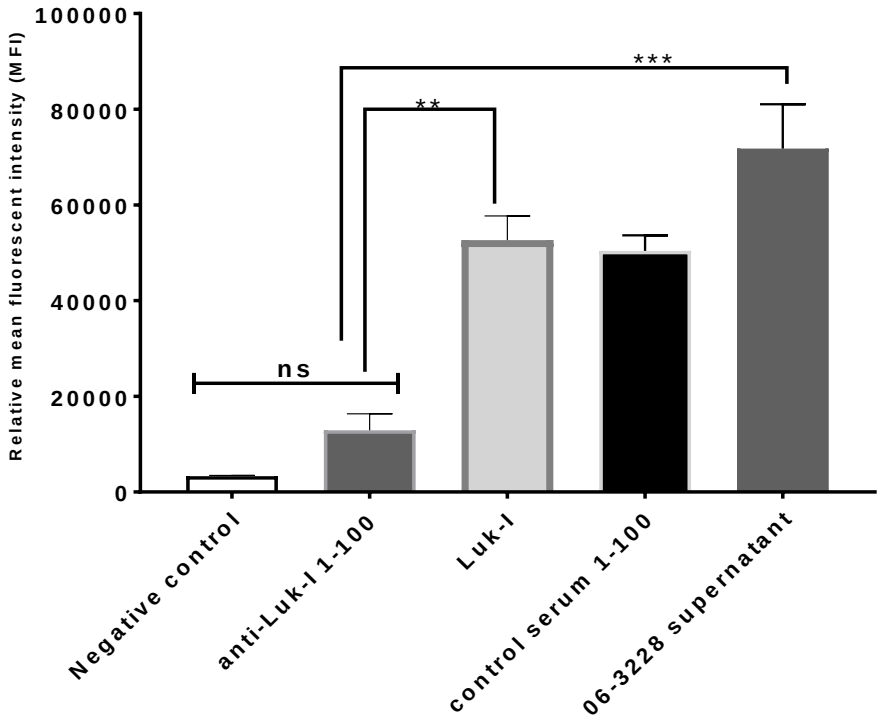

Figure 3.8 Dog antibody raised against attenuated LukS-I and LukF-I protects canine PMNs from the cytotoxic effect of Luk-I

The PMN permeability assay was performed. Luk-I preincubated with dog anti-Luk-I resulted in a significant reduction in the mean fluorescent intensity (MFI) compared with that of Luk-I treatment alone (P = 0.0036**) and with that of 06-3228 supernatant treatment alone (P = 0.0002***).

Discussion

In this study we identified a bicomponent leukotoxin, Luk-I, composed of LukS-I and LukF-I proteins secreted by *S. pseudintermedius* that kills canine PMNs by pore formation and cell lysis. Luk-I, a member of the leukocidin family, is highly conserved among *S. pseudintermedius* isolates. It is associated with an incomplete prophage that occurs in degenerate form across all *S. pseudintermedius* isolates for which genomic sequence is available.

In accordance with its host distribution in a canine opportunistic pathogen, Luk-I is cytotoxic against dog PMNs. This highlights the immune-evasive attribute of *S. pseudintermedius* Luk-I in the dog, in line with the assumed function of other phage-encoded leukocidins that similarly have a host-specific function and distribution. For example, LukMF is highly toxic to ruminant neutrophils and LukPQ preferentially kills horse neutrophils (116, 117, 135).

Based on the crystal structure of the β-sandwich (cap) domain of *S. aureus* γ-hemolysin LukF component and a *S. pseudintermedius* LukS-I and LukF-I model, we designed attenuated LukS-I and LukF-I with mutations away from the rim region. The attenuated LukS-I and LukF-I bind to canine leukocytes without causing any significant killing, indicating that mutations disrupt the ability of attenuated LukS-I and LukF-I to oligomerize, an essential function required for cytolysis. This observation is in agreement with the findings of Karauzum et al (54) with LukS-Mut9. The low concentration of endotoxin in the recombinant proteins and the low toxicity exhibited by the attenuated protein produced in *E. coli* suggests that endotoxin did not play a role in neutrophil killing.

Expression of LukF-I in *E. coli* was not successful and for unknown reasons this protein appeared to be lethal to these bacteria; therefore, a yeast protein expression system *(K. lactis)* was used for this protein. *K. lactis* is considered the best alternative to bacterial expression systems. It efficiently produces recombinant proteins in culture supernatant that are also secreted by their native host (136-138). *K. lactis* protein expression is driven by a variant of the strong PLAC4 promoter (PLAC2) (139) that lacks background expression in *E.coli* (136).

The high titer of Luk-I-specific, neutralizing antibodies produced in a clinically healthy dog injected with attenuated LukS-I and LukF-I is similar to the antibody production that Karauzum et al (54) and Adhikari et al (127) observed with LukS-PV mutant (PVL- S subunit) named “LukS-mut9” injected in mice.

With widespread antimicrobial resistance, it is critical to find alternative approaches, such as vaccines, to control staphylococcal infections. Previous studies (140-142) concluded that a multivalent vaccine would likely work best in preventing infections caused by *S. aureus*. Taking the same factors into account, a potent and effective vaccine against *S. pseudintermedius* would involve identifying antigenic targets conserved among a wide variety of strains and sequence types. Understanding the nature of extracellular proteins and their role in virulence and pathogenesis is critical for vaccine development against *S. pseudintermedius* infection.

Using mass spectrometry and genomic information, it was possible to identify a unique leucocidin present in *S. pseudintermedius* isolates 06-3221, 08-1661 and NA45, representing the three clonal complexes that predominate in the United States (82).

Conclusions

In conclusion, we describe a unique pore-forming toxin from *S. pseudintermedius*. Mutant versions of the proteins had a reduced cytotoxic effect on dog PMNs, and anti-Luk-I produced in dogs against attenuated proteins reduced the cytotoxic effect of wild type canine leukotoxin. Therefore, these mutants may serve as important components of a multivalent vaccine for prophylaxis or control of *S. pseudintermedius* infections. Such a vaccine may neutralize extracellular toxins responsible for host tissue destruction and immunosuppression and may help the host immune system control infections

CHAPTER 4
COMPLETE GENOME SEQUENCE OF *S. PSEUDINTERMEDIUS* TYPE STRAIN LMG 22219

A version of this chapter was originally published by Mohamed A. Abouelkhair, Matthew C. Riley, David A. Bemis, Stephen A. Kania:

Abouelkhair MA, Riley MC, Bemis DA, Kania SA. Complete Genome Sequence of Staphylococcus pseudintermedius Type Strain LMG 22219. Genome Announc. 2017;5(7).

No revisions were made to the original published manuscript beyond general formatting to allow consistency through the dissertation. My contributions to this paper include: (i) selection of isolates (ii) experimental design and set up (iii) experimental testing (iv) data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) writing.

We report the first complete genome sequence of LMG 22219 (=ON 86 $^{(T)}$ =CCUG 49543 $^{(T)}$), the *S. pseudintermedius* type strain isolated from feline lung tissue. This sequence information will facilitate phylogenetic comparisons of staphylococcal species and other bacteria at the genome level.

Here we present the first complete genome sequence of LMG 22219 (=ON 86 $^{(T)}$ =CCUG 49543 $^{(T)}$), the *S. pseudintermedius* type strain isolated from lung tissue of a cat (18). *S. pseudintermedius* is a Gram-positive opportunistic bacterial pathogen (143) commonly associated with canine pyoderma and occasionally isolated from human infections (144-147). It was first defined as a unique species by Devriese et al (18). This species is classified as a member of the *Staphylococcus intermedius* group which also includes *S. intermedius* and *S. delphini,* as defined by Takahashi et al. (148). *S.pseudintermedius* is characterized by non-pigmented colonies surrounded by double zone hemolysis on Columbia sheep blood agar. It is catalase-positive and coagulates rabbit plasma but is clumping-factor-negative in slide coagulase testing. *S.pseudintermedius* is positive for DNase, β-glucosidase, arginine dihydrolase, urease, nitrate reduction, pyrrolidonyl arylamidase and ONPG (β-galactosidase). It does not produce β-glucuronidase and is susceptible to 8 μg acriflavine ml −1 and to novobiocin. It is also resistant to deferoxamine (18).

Whereas assays exist to differentiate *S. pseudintermedius* from other *Staphylococcus intermedius* group species, it may be misidentified in veterinary and human medicine (147, 149). Clinical treatment of infections relies upon accurate pathogen identification (150) and the availability of the complete genome for this organism may allow for genetic validation of phenotypic testing as well as identification of new genetic targets for interspecies comparisons. Only genome sequences of *S. intermedius and S. delphini* (151) type strains are currently available and consequently the elucidation of the genome sequence of the *S. pseudintermedius* Type Strain LMG 22219 will facilitate comparisons of different species and strains based on genetic analysis.

DNA was extracted and a library prepared using the Nextera XT library preparation kit in accordance with the instructions of the manufacturer. Sequencing was performed using

Illumina MiSeq V2 (Illumina Inc., USA) and De novo assembly was performed using Geneious v.9.1.6 (107). Automated annotation of assembled contigs was performed using the NCBI Prokaryote Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok).

A total of 1,432,416 MiSeq paired-end reads were used to generate 50 contigs with > 100 x average depth of coverage. The LMG 22219 genome is comprised of 2,523,112 base pairs with 37.6% GC content, 2,242 predicted coding sequences and 81 RNAs.

Accession numbers. This whole-genome has been deposited at DDBJ/ENA/GenBank under the accession no. MLGE00000000. The version described in this paper is version MLGE00000000.1.

Acknowledgments

We thank the University of Tennessee Institute of Agriculture Center of Excellence in Livestock Diseases and Human Health for funding and support.

CHAPTER 5
IDENTIFICATION, CLONING AND CHARACTERIZATION OF SPEX EXOTOXIN PRODUCED BY *S. PSEUDINTERMEDIUS*

A version of this chapter was submitted to a scientific Journal for publication. No revisions were made to the original published manuscript beyond general formatting to allow consistency through the dissertation. My contributions to this paper include: (i) selection of isolates (ii) experimental design and set up (iii) experimental testing (iv) data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) writing.

Abstract

Staphylococci have evolved numerous strategies to evade the immune system of their hosts. Some staphylococcal toxins target essential components of host innate immunity, one of the two main branches of the immune system. Analysis of the *S. pseudintermedius* secretome using liquid chromatography mass spectrometry guided by genomic data, was used to identify an *S. pseudintermedius* exotoxin provisionally named SpEX. This exoprotein has low overall amino acid identity with the *Staphylococcus aureus* group of proteins named staphylococcal superantigen like proteins (SSLs) and staphylococcal enterotoxin- like toxin X (SEIX), but predictive modeling showed that it shares similar folds and domain architecture to these important virulence factors. In this study, we found SpEX binds to complement component C5, prevents complement mediated lysis of sensitized bovine red blood cells, kills polymorphonuclear leukocytes and monocytes and inhibits neutrophil migration at sub-lethal concentrations. A mutant version of SpEX, produced through amino acid substitution at selected positions, had diminished cytotoxicity. Anti-SpEX produced in dogs reduced the inhibitory effect of native SpEX on canine neutrophil migration and protected immune cells from the toxic effects of the native recombinant protein. These results suggest that SpEX likely plays an important role in *S. pseudintermedius* virulence and that attenuated SpEX may be an important candidate for inclusion in a vaccine against *S. pseudintermedius* infections.

Introduction

S. pseudintermedius is the main cause of canine dermatological disease and has been isolated from wound and surgical site infections, endocarditis and mastitis in dogs (6, 18). Human infections with this organism have been reported sporadically, most of which have been related to exposure to dogs (80). As many as 30-40% of the *S. pseudintermedius* isolates tested in clinical laboratories in different geographical areas are methicillin-resistant (MRSP) (7). Most MRSP are multidrug-resistant leaving few treatment options. The transfer of resistance genes to human organisms as well as direct transfer of multidrug-resistant *S. pseudintermedius* between animals and to humans are theoretical concerns (82-85). Development of alternative approaches to control staphylococcal infections, such as vaccines, is challenging. *S. pseudintermedius* may evade the immune

response of their hosts by producing a number of cell surface and secreted proteins that target essential components of the host defense, promoting the survival and transmission of this pathogen. *S. pseudintermedius,* like *Staphylococcus aureus*, produces hemolysins, exfoliative toxins, leukotoxins, thermonuclease, protein A, coagulase and adenosine synthase (13, 15-17, 21). However, many of these virulence factors are not well characterized in *S. pseudintermedius* and others likely remain to be identified.

Innate immunity involving neutrophils is an important first step in the defense against staphylococci and migration from blood provides the majority of these effectors. This process is triggered by inflammatory signal molecules such as C5a, neutrophils bind to upregulated endothelial selectins, extravasate and migrate toward the site of inflammation (63). However, neutrophils are susceptible to staphylococcal defenses including molecules that inhibit neutrophil function and leukotoxins that kill by forming pores in their cell membranes (152) .

The roles of structurally related *S. aureus* secreted virulence factors staphylococcal superantigen-like proteins (SSLs) and staphylococcal enterotoxin-like toxin (SEIX) in inhibiting the innate immune response of their hosts are well characterized. SSLs are distinguished from SEIX by their lack of cytotoxic activity. SSLs are two- domain proteins with an average size of 25 kDa (64, 65). The first domain, located at the N-terminus, displays an oligosaccharide/ oligonucleotide binding (OB) fold forming a β- barrel. The second domain possesses a β-grasp motif consisting of a twisted β-sheet of four to five antiparallel strands, located at the C-terminus. The two domains are separated by a structurally conserved α-helix. SSLs target innate immunity components but do not bind to T cell receptors or the major histocompatibility complex (67, 153).

In previous studies, *S. aureus* SSL4 (67), SSL5 (65) and SSL11 (65), were found to interfere with neutrophil migration through their sialated glycan-binding site in the C-terminal β-grasp domain. Chung et al., (68) showed that SSL11 with a single site mutation, T168P, had defective binding compared to native SSL11 and lost its inhibitory effect on neutrophil attachment to P-selectin. The OB fold domain of SSL7 binds to the IgA Fc region while the β-grasp motif adheres tightly to complement component C5 resulting in inhibition of complement mediated hemolytic activity (65). SSL3 binds to and

inhibits toll-like receptor (TLR) 2 using a well characterized recognition site not found in other SSLs (118). Tuffs et al., (66) showed that SEIX secreted by *S. aureus* shares its domain structure with SSLs. As with SSLs, SEIX binds to neutrophils, however, it also exhibits a superantigenic effect via its OB fold on T cells (66, 67).

In this study, we used a proteomic approach to identify an exoprotein produced by *S. pseudintermedius*, SpEX. It is most closely related to and shares some biological properties and domain structures with SSLs and SEIX, however, it has less than 50% amino acid similarity with SSL proteins and has key distinguishing properties. It is often annotated in *S. pseudintermedius* as exotoxin 15, a synonym for SSL11, formerly referred to as staphylococcal exotoxin-like proteins (SET) (154). SpEX was studied to determine if it had immunosuppressive effects on the innate immune system of their hosts. The specific objectives of this study were to characterize the immunobiological properties of *S. pseudintermedius* SpEX and attenuated (reduced toxicity) SpEX (SpEX-M) by measuring their inhibitory effects on complement activity, neutrophil migration and cytotoxic effects on PMNs and monocytes. Antibody against SpEX-M was developed in clinically healthy dogs and the neutralizing activity of those antibodies was evaluated.

Results

Identification of putative immune modulator from *S. pseudintermedius*

Liquid chromatography–mass spectrometry (LC-MS/MS) was used to screen culture supernatants of three clinical strains of *S. pseudintermedius* (06-3228, 08-1661 and NA45), representing the major *S. pseudintermedius* genotypes occurring in the United States as determined by multilocus sequence typing (8). We identified over 500 secreted or cell wall associated proteins and others with unknown cellular locations (manuscript under review). The secretome proteins were compared among 06-3228, 08-1661 and NA45 strains using their respective genomes as references. SpEX, a 234-aa protein, was detected in the supernatants of the three isolates. The mean predicted molecular weight was 26.093 kDa with a mean isoelectric point of 6.30.

Bioinformatics analysis of *S. pseudintermedius* SpEX

A BLAST search of SpEX in the GenBank database revealed that it is conserved among *S. pseudintermedius* strains including 06–3221, 08–1661 and NA45 with amino acid identities over 88% between strains. SpEX shares sequence similarity with other pathogenic bacterial species within the *Staphylococcus intermedius* group. It shares amino acid identity of 71.8% with *Staphylococcus intermedius,* 73.5% with *Staphylococcus delphini* and 72.2% with *Staphylococcus cornubiensis.* No similar proteins were identified in *S. aureus*. SpEX has approximately 47% amino acid sequence identity with *S. aureus* SSL11 and less than 30% amino acid identity with other SSL members and SEIX.

Analysis of SpEX secondary and tertiary domain structures using Geneious 11.0.3 software (107) supported by a SpEX model developed with the Phyre^2 web portal showed that *S. pseudintermedius* SpEX share fold and domain architecture with *S. aureus* SSLs and SEIX (**Figure 5.1, a and b**). The SpEX protein sequence contains a signal peptide sequence from positions 1 through 35 detected using SignalP 4.1 (134). SpEX has an N-terminal OB domain in residues 43-126 that folds into a five-stranded beta-barrel structure consisting of β-strands (β1- β5) and a C-terminal β grasp motif in residues 150-234 (**Figure 5.1, a**). This comprises a β sheet formed from β7, β6, β12, β9 and β10 with a characteristic central helix orientated diagonally in the center of the field from the top left to the bottom right leaving the N-terminal domain on the left and C-terminal domain on the right (**Figure 5.1, b**).



Figure 5.1 *S. pseudintermedius* SpEX structural characteristics (a) SpEX of *S. pseudintermedius* harbors an N-terminal signal peptide (green arrow) from position 1-35, OB domain (orange arrow) in residues 43-126 and C-terminal β grasp domain in residues 150-234 (orange arrow).

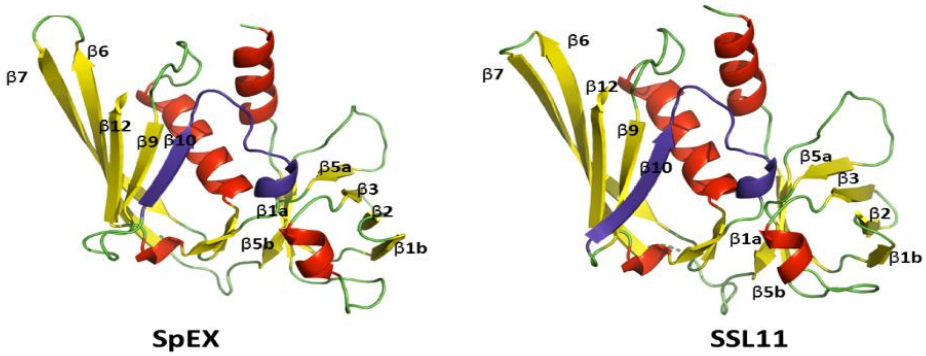

Figure 5.1(b) The 3D model of *S. pseudintermedius* SpEX and *S. aureus* SSL11 shows the N and C terminal domains fold into a five-stranded beta-barrel structure consisting of β1- β5 and β7, β6, β12, β9 and β10. A central helix is orientated diagonally in the center of the field from the top left to the bottom right leaving the N-terminal domain on the left and C-terminal domain on the right. A conserved sialated glycan binding domain forming V-shape depression is highlighted in purple in SpEX and SSL11 3D models.

S. aureus SSL4 (accession number: WP_000705627.1), SSL5 (accession number: WP_000784244.1), SSL11 (accession number: WP_000769163.1) and SEIX (accession number: AEI60186.1) proteins have been shown to bind sialated glycan through a conserved sialated glycan binding domain located at the C-terminal β grasp domain (67, 68, 155). It has the capacity to influence the innate immunity defenses through targeting sialated glycoproteins at the surface of immune cells. Amino acid multiple sequence alignment of SpEX with previously characterized *S. aureus* SSL4, SSL5, SSL11 and SEIX proteins showed that *S. pseudintermedius* SpEX possesses the conserved sialated glycan binding site of the SSLs located at the C-terminal region and forming the V-shape depression in the 3D model of SpEX (**Figure 5.2**).

In *S. pseudintermedius* SpEX, we predict aspartic acid (D) 102 and threonine (T) 125 in the OB- fold domain are critical residues for the cytotoxic effect of the protein on immune cells. T 206, serine (S) 208, lysine (K) 211, leucine (L) 213, glutamine (Q) 214, arginine (R) 217 and isoleucine (I) 227 lining the sides of the glycan binding domain were predicted

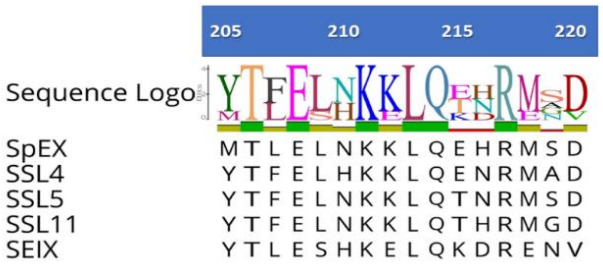

Figure 5.2 Multiple sequence alignment of SpEX glycan binding domain with *S. aureus* SSL and SEIX. Proteins with accession numbers are SSL4 (WP_000705627.1), SSL5 (WP_000784244.1), SSL11 (WP_000769163.1) and SEIX (AEI60186.1). Threonine (T) 206, serine (S) 208, lysine (K) 211, leucine (L) 213 and arginine (R) 217 are identical among all analyzed proteins (five proteins). The numbers refer to the position of the amino acids in full length SpEX protein.

as essential for binding to monocytes, neutrophils and complement. These residues are highly conserved among SSLs and SEIX proteins.

Design of SpEX with amino acid substitutions

An attenuated *S. pseudintermedius* SpEX (SpEX-M) was designed with (D102A and T125P) in the OB-fold domain and (T206P and R217A) in the sialated glycan binding domain (**Figure 5.3**).

To ensure that protein stability and folding were not altered, only amino acids highly conserved within functional domains of *S. pseudintermedius* SpEX and *S. aureus* SSL4, SSL5, SSL11 and SEIX were selected. Furthermore, the designed SpEX was first modelled in the structure to confirm that its conformation was maintained (**Figure 5.3**).



Figure 5.3 Residues substituted to produce attenuated *S. pseudintermedius* SpEX-M

Pairwise amino acid sequence alignment between recombinant SpEX and SpEX-M. Attenuated SpEX-M had substitutions of D102A and T125P (highlighted in purple and orange color, respectively) in the OB-fold domain and T206P and R217A (highlighted in green and yellow color, respectively) in the sialated glycan binding domain. The herpes simplex virus (HSV) tag and 6x his tag was annotated with red arrows whereas the protein domains were annotated with orange arrows.

Cloning, expression, and purification of recombinant *S. pseudintermedius* SpEX

Recombinant native and mutant SpEXs with C- terminal 6x histidines (**Figure 5.3**) were generated in *E. coli* and purified under native conditions using HisPur Ni-NTA affinity chromatography. Both SpEX and SpEX-M expressed well from *E. coli* and remained soluble in solution, indicating protein stability with the amino acid substitutions. The molecular weights of SpEX and SpEX-M determined in western blots were of the expected sizes (27. 63 and 27.49 kDa, respectively) (**Figure 5.4**).

SpEX interferes with complement function

Binding of SpEX to human complement C5 was detected using ELISA, wherein complement component C5 was coated on ELISA plates and binding of recombinant native and attenuated his-tagged SpEX to C5 was measured using horseradish

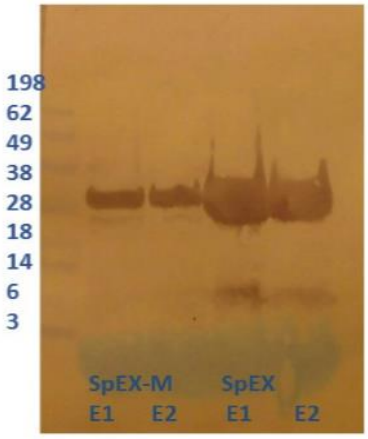

Figure 5.4 Western blot of recombinant *S. pseudintermedius* native and mutant SpEX detected with HRP-conjugated anti-6xhis

The Molecular weights of recombinant SpEX (rSpEX) and rSpEX-M, after expression in Tuner ™ (DE3) pLacI *E. coli* induced by Isopropyl β-D-1-thiogalatopyranoside (IPTG), determined in western blots were of the expected sizes (27. 63 and 27.49 kDa, respectively) in the elution fractions (E1 and E2).

peroxidase (HRP)-conjugated anti-his tag monoclonal antibody. SpEX bound to human C5 significantly higher, in a dose dependent manner (0.5 with P =0.0171, 1 µg /ml with P =0.0027, 2 and 4 µg /ml with P <0.0001), than SpEX-M (**Figure 5.5, a**).

A hemolytic assay was used to evaluate SpEX mediated- inhibition of RBC lysis by complement. SpEX fixed complement and caused inhibition of hemolysis in a concentration dependent manner. At concentrations of 0.5, 1, 2 and 4 µg /ml, SpEX reduced the hemolysis of sensitized bovine erythrocytes compared to the positive control with P < 0.0001. SpEX at a concentration of 4 µg/ml showed no significant difference in hemolysis compared with the negative control (**Figure 5.5, b**)

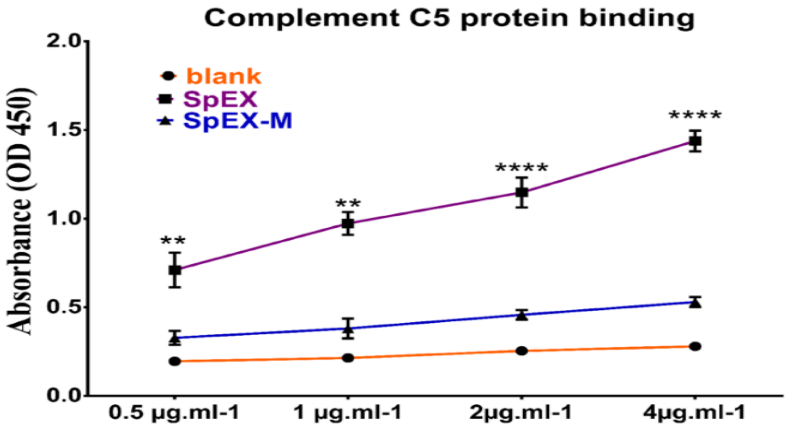

Figure 5.5 SpEX interferes with complement function

(a) HRP-conjugated anti-6xhis tag monoclonal antibody was used at a dilution of 1/1000 to detect recombinant SpEX bound to human C5. Recombinant SpEX bound significantly higher to human C5 than recombinant SpEX-M protein (0.5 with P =0.0171**, 1 µg /ml with P =0.0027**, 2 and 4 µg /ml with P <0.0001****). These values represent averages from three independent experiments. (*P < 0.05 was considered significant).

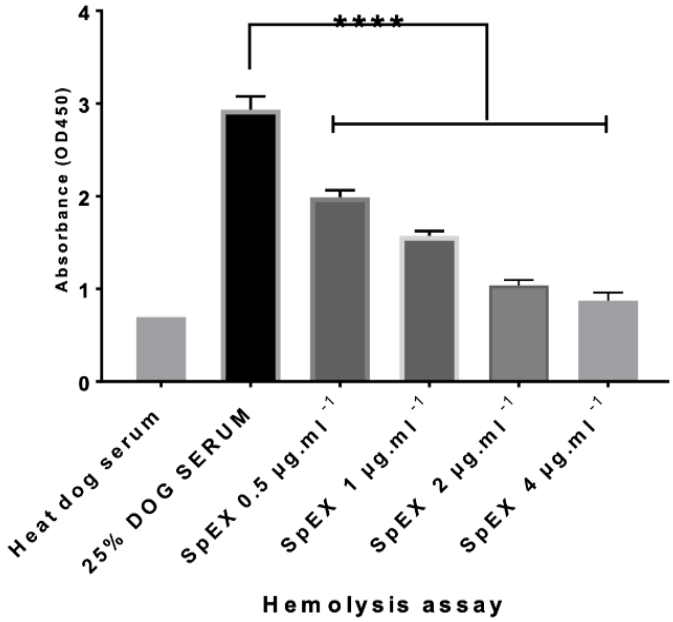

Figure 5.5 (b). Starting at a concentration of 0.5 µg/ml, SpEX significantly reduced the hemolysis of sensitized bovine erythrocytes compared to the positive control with P < 0.0001****. SpEX at a concentration of 4 µg/ml showed no significant difference in hemolysis compared with the negative control. The values represent averages from three independent experiments. (*P < 0.05 was considered significant).

SpEX inhibits neutrophil migration

A trans-well neutrophil migration assay was used to determine the inhibitory effect of recombinant SpEX on neutrophil migration *in vitro*. Recombinant SpEX at 0.2 µg/ml (at a concentration selected to assure cells remained viable (**Figure 5.8**) inhibited the migration of neutrophils induced by fetal bovine serum compared to SpEX-M at 0.2 µg /ml with P =0.0001 (**Figure 5.6**). To validate the results, the viability of neutrophils during experiments was examined to ensure that killing did not occur at the concentration of SpEX used.

Attenuated SpEX induces a strong antibody response

Recombinant SpEX-M at 20 µg in 0.5 ml in phosphate buffered saline (PBS) (pH 7.2) was injected into three clinically normal dogs subcutaneously in the lateral thorax.

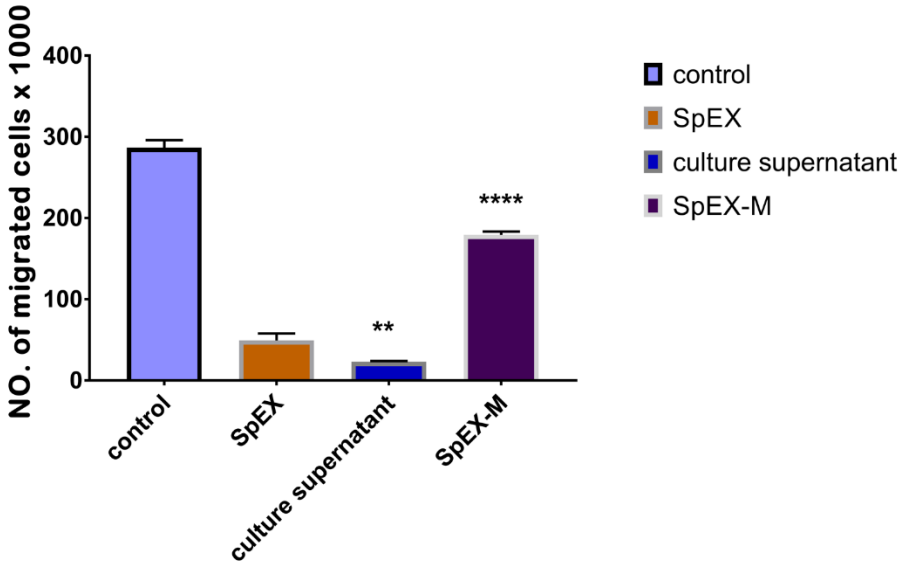

Figure 5.6 PMN transmigration assay

Recombinant SpEX at a concentration of 0.2 µg /ml significantly inhibited the migration of PMNs induced by fetal bovine serum compared to SpEX-M at the same concentration with P < 0.0001****. The chemotaxis inhibition by culture supernatant of *S. pseudintermedius* 06-3228 was significantly higher than SpEX P = 0.0035**. The values represent averages from three independent experiments. (*P < 0.05 was considered significant). Data are plotted on the Y axis X 1000.

Sera were collected from dogs on days -7, 8, 15 and 29 (relative to SpEX-M injections). IgG reactive with SpEX-M and SpEX was detected by ELISA on day 15 ($P < 0.0001$) and at a higher level on day 29 ($P < 0.0001$) compared to pre-injection control sera (**Figure 5.7**). Both SpEX and SpEX-M were recognized by dog anti-SpEX-M, confirming that there were no major antigenic differences between native and mutant SpEX.

SpEX kills canine PMNs and monocytes

Canine PMNs and monocytes harvested from canine blood were highly susceptible to SpEX with cell permeability induced within 30 minutes in a concentration dependent manner (50, 25, 12.5, 6.25, 3.12 µg SpEX/ml in PBS, pH 7.2) and by a 1:2 dilution of *S. pseudintermedius* strain 06-3228 culture supernatant (**Figure 5.8**). SpEX-M showed a diminished effect on cell permeability of canine PMNs ($P = 0.0052$) (**Figure5.8**)

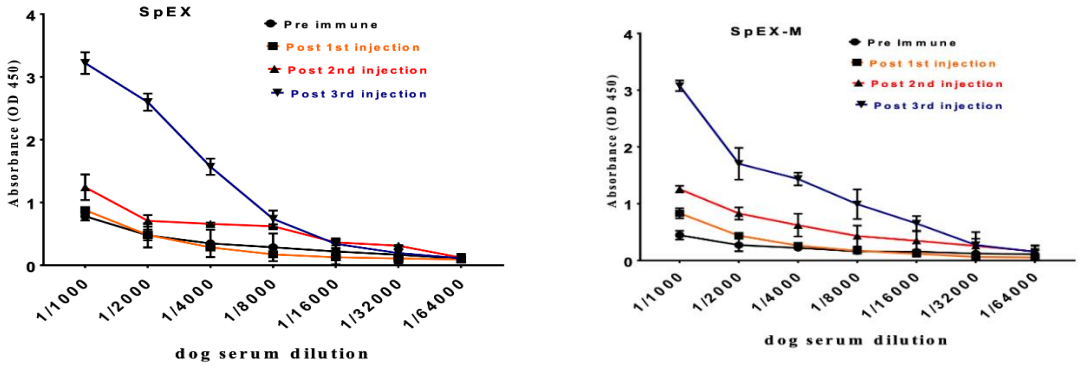

Figure 5.7 Dogs injected with SpEX-M developed IgG specifically reactive with recombinant native SpEX and SpEX-M

Antibodies against *S. pseudintermedius* native SpEX and SpEX-M were measured using an indirect ELISA. Recombinant *S. pseudintermedius* SpEX and SpEX-M proteins were coated on ELISA plates, then incubated with two-fold serially diluted serum from dogs vaccinated with the same proteins. High reactivity with SpEX and SpEX-M occurred with sera collected two weeks after the third injections of SpEX-M ($P=0.0001$) compared to pre-injection sera. The values represent averages from three independent experiments.

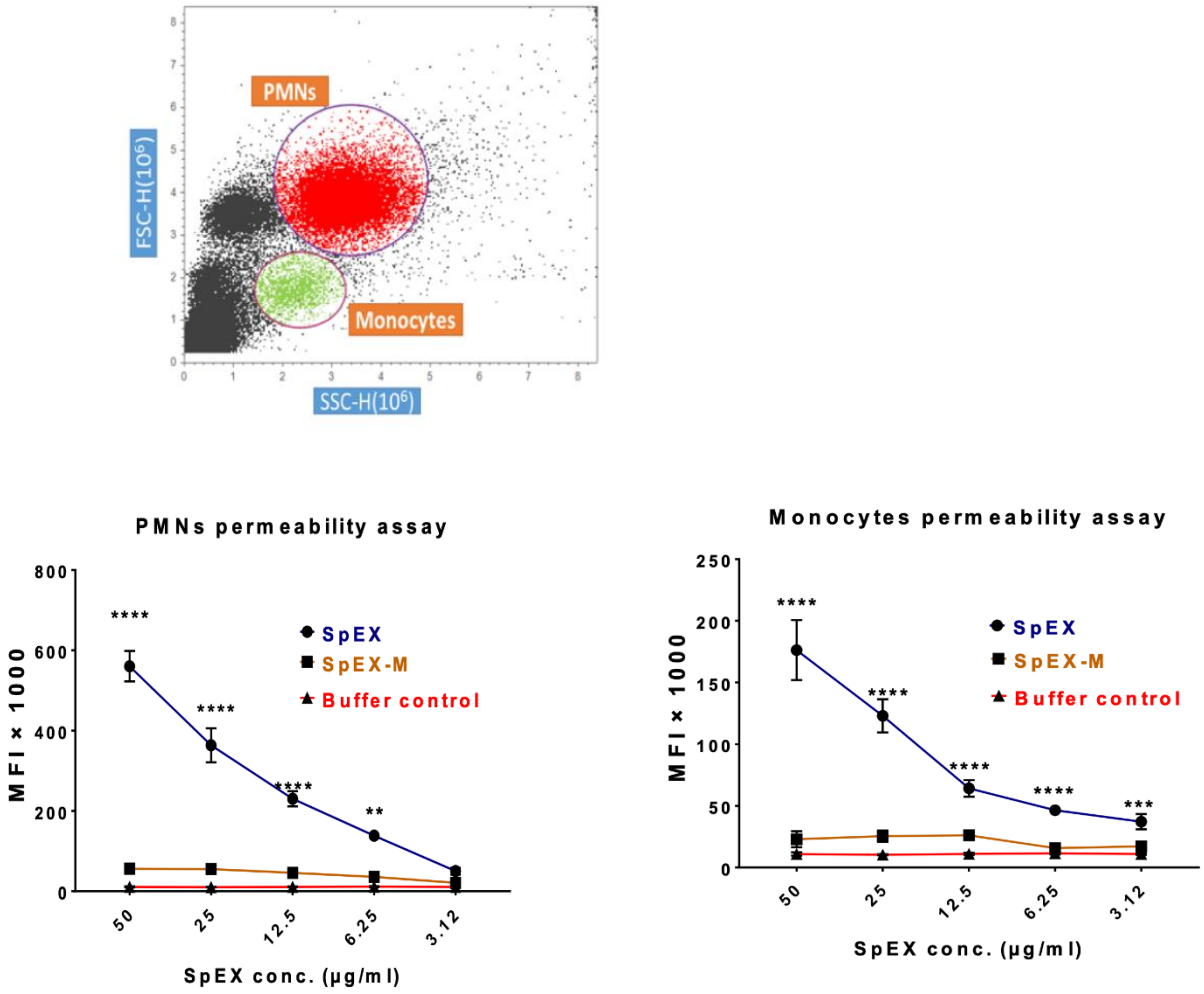

Figure 5.8 *S. pseudintermedius* recombinant SpEX has a cytotoxic effect on canine monocytes and PMNs

Gating on canine monocytes (green color) and PMNs (red color) was based on side and forward scatter (shown in dot plot). The mean fluorescent intensity (MFI) of the buffer control and SpEX-M relative to SpEX was significantly lower in PMN ($P < 0.0001$****) and monocyte ($P < 0.0001$****) permeability assays.

The values were calculated based on average values from three independent experiments (*$P < 0.05$ was considered significant, **** $P < 0.0001$, **** $P = 0.0043$).

and monocytes compared to SpEX (P < 0.0001) (**Figure 5.8**). At SpEX concentrations of 50, 25, 12.5, 6.25 and 3.12 µg/ml, monocyte cell permeability was significantly different from that of SpEX-M and the negative control (P = 0.0001) (**Figure 5.8**). However, SpEX at concentrations of 50, 25, 12.5 and 6.25 µg/ml, had a significant effect on PMN cell permeability compared to SpEX-M and negative control (P =0.0001) (**Figure 5.8**).

Canine anti-SpEX-M reduced the effects of SpEX on PMNs and monocytes *in vitro*

Canine anti-SpEX-M collected after 3rd injection, at a dilution of 1:100 in PBS (pH 7.2), preincubated with recombinant SpEX at a concentration of 0.2 µg /ml, significantly reduced the inhibitory effect of rSpEX on leukocyte chemotaxis compared to serum collected from dogs before SpEX-M injection and control serum with P < 0.0001 (**Figure 5.9, a**).

In a measure of the protective effect of canine anti-SpEX-M against the native protein, rSpEX (3.1 µg per ml of PBS) treated with anti-SpEX-M at dilution 1:100 significantly reduced cell permeability represented by mean fluorescent intensity (MFI) as compared with that of SpEX treatment alone (**Figure 5.9, b**)

Discussion

Using mass spectrometry referenced to genomic data, it was possible to identify a new *S. pseudintermedius* exotoxin, SpEX, in *S. pseudintermedius*. SpEX secretion was confirmed in three *S. pseudintermedius* strains representing the three clonal complexes that predominate in the United States (82). SpEX from the most commonly isolated clonal complex, CC68, was used in this study. However, SpEX is highly conserved among all three clonal complexes. Although its sequence is unique, this protein has a typical SSL tertiary structure, shared by *S. aureus* SSLs and SEIX, consisting of an N-terminal OB-fold domain that folds into a five-stranded β-barrel and a C-terminal β-grasp domain. However, SpEX, in addition to sharing the chemotaxis and complement inhibitory properties of SSLs, has a cytotoxic effect against monocytes and PMNs

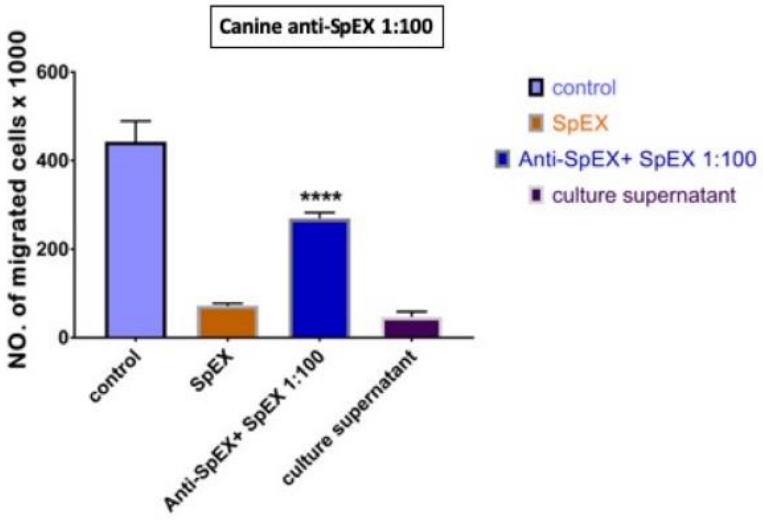

Figure 5.9 Canine anti-SpEX-M reduced the effects of SpEX on PMNs and monocytes in vitro

(a) Canine anti-SpEX, collected after three injections, at a dilution of 1:100 in PBS (pH 7.2), preincubated with recombinant SpEX, significantly diminished the chemotaxis inhibition of SpEX with $P < 0.0001$****.

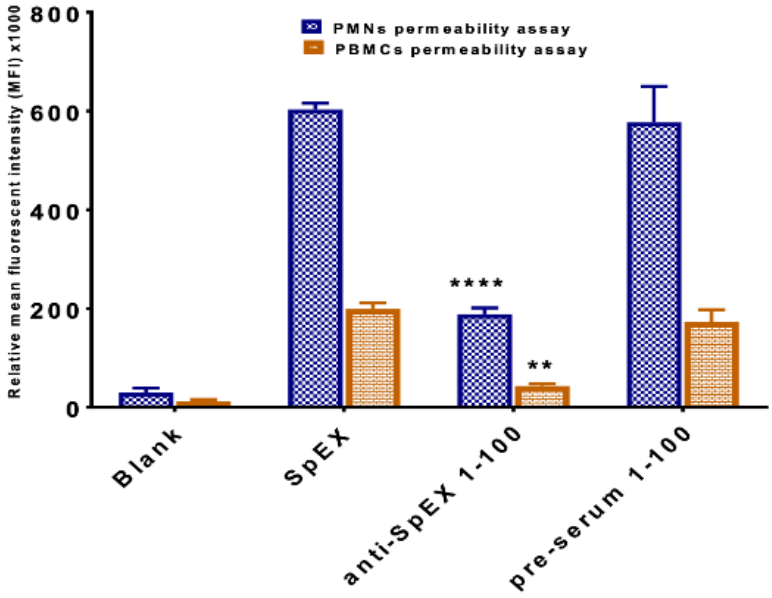

Figure 5.9 (b). Pre-incubation of canine anti-SpEX diluted 1:100 with SpEX resulted in a significant reduction in MFI as compared with that of SpEX treatment alone. There was no significant difference between SpEX and SpEX treated with pre-serum. The MFI of SpEX pre-incubated with anti-SpEX 1:100 relative to SpEX was significantly lower in PMN ($P < 0.0001$****) and monocyte ($P = 0.012$**) permeability assays. The values calculated were based on average values from three independent experiments (*$P < 0.05$ was considered significant).

These functions likely promote bacterial survival in their hosts and increase the likelihood of transmission. The attenuated SpEX remained soluble following expression from *E. coli* and both SpEX and SpEX-M were recognized by dog anti-SpEX-M. Thus, SpEX-M with amino acid substitutions in its predicted functional domains did not induce significant antigenic or conformational changes in the protein, however it showed diminished immune evasive properties compared to SpEX.

Antibody-mediated toxin neutralization may provide a strategy for immunotherapeutic treatment and/or prevention of current and recurrent infection (116, 119). Canine antibodies to SpEX neutralize and diminish its chemotactic inhibitory and cytotoxic effects on PMNs and monocytes highlighting the potential value of this protein as a component in a vaccine to prevent or treat *S. pseudintermedius* infections.

The increased prevalence of multidrug resistant *S. pseudintermedius leaves few* options for antimicrobial therapy (82). Therefore, the development of novel strategies to treat this pathogen are a research priority (82, 142). One alternative approach is the development of a vaccine that can confer protection or provide effective therapy. However, vaccines in *S. aureus* clinical trials have thus far failed to show efficient protection (156). Previous studies (140-142) concluded that multicomponent vaccines containing a cocktail of staphylococcal antigens would likely work best in preventing infections caused by *S. aureus*. We propose that a potent and effective vaccine against *S. pseudintermedius* would involve immunogenic targets, secreted and/or accessible on the surface of the bacterium, conserved among prevalent strains and that play important roles in virulence, such as immune evasion. Understanding *S. pseudintermedius* protein function, surface accessibility and epitope conservation is crucial for vaccine development against infections caused by this pathogen.

In conclusion, we describe a new exotoxin produced by *S. pseudintermedius* that binds to C5, inhibits complement activation and permeabilizes leukocytes. A mutant version of the protein has reduced cytotoxic effects on dog PMNs and monocytes *in vitro*. Canine anti-SpEX produced against an attenuated form of the protein reduced its chemotaxis inhibition. Therefore, these mutant proteins may serve as important components of a multivalent vaccine for prophylaxis of *S. pseudintermedius* infections. By neutralizing

extracellular toxins responsible for host tissue destruction and immunosuppression, such a vaccine may help the host immune system control infections.

Future studies might examine potential interactions with TLR as well as regulation of *SpEX* expression and the effects of quorum sensing and biofilm on production of the protein to better understand its role in pathogenesis. In addition, orthologs of SpEX produced by other members of the *Staphylococcus intermedius* group should be studied to determine their role in the virulence of these species.

Materials and Methods

Ethics Statement

Experimental protocols were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC) including 2474-0716 for samples of dog blood and 2572-1217 for producing antibodies in dogs with recombinant protein.

Bacterial strains and plasmids

Log phase bacterial cultures of *S.pseudintermedius* strains (shown with their sequence types) 06-3228 (ST68), 08-1661 (ST71) and NA45 (ST84) were analyzed by mass spectrometry. These strains are representative of the major *S. pseudintermedius* genotypes (clonal complexes) most commonly isolated from canine infections in the United States. A plasmid construct containing a mutated, synthetic *S. pseudintermedius spEX* (designed as described below) with BamHI/NotI cloning sites, was obtained commercially (Genscript Piscataway, NJ USA) (**Table 5.1**).

Media and growth conditions

Bacterial colonies grown on blood agar plates were inoculated into 5ml of trypticase soy broth (TSB) (BD Biosciences, San Jose, CA) at 37°C with shaking at 225 rpm. For log-phase bacterial cultures, bacteria were grown until an optical density (OD_{600}) of 0.4-0.6 was reached.

Table 5.1 Bacteria, plasmids and competent cells used in this study

Plasmid/ Bacteria	Characteristics	Source
pUC19-SpEX-M construct	Cloning plasmid containing synthetic, attenuated *S. pseudintermedius* SpEX (717 bp) inserted into MCS of pUC19 between BamHI and NotI restriction sites	Genscript Piscataway, NJ USA
pETBlue-2	Plasmid for T7 promoter based expression of recombinant proteins with blue/white screening and C-terminal HSV•Tag and His•Tag sequences	Novagen, Madison, WI
pETBlue-2 SpEX construct	Plasmid containing synthetic *S. pseudintermedius* SpEX (716 bp) inserted into MCS of pETBlue-2 between BamHI and NotI restriction sites and C-terminal His•Tag sequences	This study
pETBlue-2 SpEX-M construct	Plasmid containing synthetic *S. pseudintermedius* SpEX-M (717 bp) inserted into MCS of pETBlue-2 between BamHI and NotI restriction sites and C-terminal His•Tag sequences	This study
DH5α™ *E.coli*	Competent cells used for cloning pETBlue-2 constructs. Genotype: F^- Φ80*lac*ZΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-.	Novagen, Madison, WI
Tuner™(DE3) pLacI *E.coli*	lacZY deletion mutants of BL21 and a lysogen of λDE3 that carries a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter.	Novagen, Madison, WI
***S. pseudintermedius* 06-3228**	Strain representing the most common ST in the United States (ST68)	*
***S. pseudintermedius* 08-1661**	Strain representing the most common ST in Europe and among the three most common in the United States (ST71)	*
***S. pseudintermedius* NA45**	Strain representing the most common ST in Asia and among the three most common in the United States (ST84)	**

*University of Tennessee, College of Veterinary Medicine Bacteriology Laboratory.

** A gift of Faye Hartmann of the University of Wisconsin, School of Veterinary Medicine.

LC-MS/MS analysis of *S. pseudintermedius* supernatant

Log phase bacterial cultures of 06-3228, 08-1661 and NA45 were centrifuged at 10,000g for 30 minutes at 4°C and the supernatants were collected and passed through 0.45µm filters (Whatman, GE Healthcare Lifesciences, Pittsburgh, PA). The filtrates were concentrated using Amicon® ^{Ultra}-4 centrifugal filters (EMD Millipore Corp., Billerica, MA) and stored at -20°C until further analysis. Samples interrogating *S. pseudintermedius* supernatant were prepared for shotgun LC-MS/MS analysis. Trypticase soy broth (media alone) and bacterial culture supernatant passed through control Sepahrose beads (without IgG) served as controls. Peptides were separated and analyzed with a 2-step MudPIT LC-MS/MS protocol (salt cuts of 50mM and 50mM ammonium acetate) over a 4-hr period then measured with a hybrid LTQ XL-Orbitrap (Thermo Scientific, Waltham, MA) mass spectrometer (MS). The percent coverage of detected proteins was calculated as previously described (157).

Bioinformatics analysis

Multiple sequence alignment (MSA) of SpEX proteins from a total of 123 *S. pseudintermedius* isolates available in the Genbank database and others sequenced previously in our lab (7-9) was performed using Geneious 11.0.3 software (107). Protein Homology/analogY Recognition Engine V 2.0 (Phyre^2) was used to develop a *S. pseudintermedius* SpEX model (http://www.sbg.bio.ic.ac.uk/phyre^2) with alignment coverage, identity percent and confidence set to 82%, 100% and 40% respectively (109). The 3DLigandSite online tool (http://www.sbg.bio.ic.ac.uk/3dligandsite/) was used for binding site prediction (110).

The *S. pseudintermedius* SpEX sequence was aligned to *S. aureus* SSL4, SSL5, SSL11 and SEIX to identify the shared protein domain and secondary structures. We designed a full-length, attenuated *S. pseudintermedius* SpEX construct (SpEX-M), with the following amino acid substitutions: D102A, T125P, T206P and R217A.

Polymerase chain reaction (PCR) amplification of *SpEX*

DNA was extracted using a MO BIO UltraClean® Microbial DNA Isolation Kit (QIAGEN Inc.) according to the manufacturer instructions. Oligonucleotide primers (Integrated DNA Technology, Coralville, USA) (**Table 5.2**) were designed using the PrimerQuest

Table 5.2 Primers used to amplify recombinant wild- type and attenuated spEX from *S. pseudintermedius* and pUC19-spEX-M plasmid

NotI and BamHI restriction sites are bold and underlined, nucleotide sequences belonging to SpEX and SpEX-M are show in italics, the primer binding sites are highlighted in purple color and sequences outside SpEX and SpEX-M (GCATGA) were used to flank NotI and BamHI recognition sites.

Primer	Sequence
SpEX forward	GCATGA**GGATCC**A*AGCGAAGCACATGCCCA*
SpEX reverse	GCATGA**GCGGCCGC***CAGATCTATCGTAATTTGTTGGA*
SpEX-M forward	**GCATGA**GGATCC*AATGAAACAGAGCACCATTCT*
SpEX-M reverse	**GCATGA**GCGGCCGC*CAGGTCGATG*

Tool (https://www.idtdna.com/Primerquest/Home/Index) based on the genomic sequence of *S. pseudintermedius* strain 06-3228 (9).

The native *SpEX* open reading frame (ORF) (705bp) excluding N-terminal signal peptide was amplified from *S. pseudintermedius* strain 06-3228 genomic DNA and the mutant *SpEX* was amplified from a pUC19-*spEX*-M plasmid (Genscript Piscataway, NJ USA) (**Table 5.1**) using taq polymerase (rTaq, Takara).

Cloning, expression, and purification of recombinant native and attenuated SpEX

S. pseudintermedius native and mutant *SpEX* PCR products were cloned using NotI and BamHI digested pETBlue-2 (Novagen,) (**Table 5.1**). The plasmid constructs were transformed into cloning host DH5-alpha *E. coli* chemically-competent cells, (**Table 5.1**) (New England BioLabs Inc.,) before being transformed into expression host, Tuner ™ (DE3) pLacI *E. coli* chemically-competent cells (**Table 5.1**) (Novagen,) by heat shock. Recombinant *S. pseudintermedius* native and mutant SpEX expression were performed from *E. coli* pETBlue-2 constructs as previously described (17) using LB broth containing 50µg/ml ampicillin and 20µg/ml chloramphenicol as protein expression

media. Protein expression was induced by addition of 1 mM Isopropyl β-D-1-thiogalatopyranoside (IPTG). Protein extraction was performed using BugBuster reagent (Novagen) and 100X protease inhibitor (Cocktail Set III, EDTA-Free Calbiochem,). Recombinant proteins were purified using affinity purification (HisPur™ Ni-NTA Spin Purification Kit, Thermo Scientific). Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Scientific).

SDS-PAGE and western blot

SDS-PAGE in 4-12 % polyacrylamide gels (Invitrogen) was used to measure the molecular weight of expressed recombinant proteins. The resolved protein samples were blotted onto nitrocellulose membranes (Thermo Scientific). Then the blots were incubated in 5% (wt/vol) nonfat dried milk powder in 0.05% polyethylene glycol sorbitan monolaurate (Tween 20) containing phosphate buffered saline (PBS-T) overnight at 4°C. Horseradish peroxidase (HRP)-conjugated anti-6xhis tag monoclonal antibody (Thermo Scientific) diluted 1:2,000 in 0.05% PBS-T was added to the blocked membranes and incubated for 1 h with 225 rpm shaking at room temperature. After five washes with 0.05% PBS-T, bound antibodies were detected using 1-Step™ chloronaphthol substrate solution (Thermo Scientific).

Preparation of canine anti-*S. pseudintermedius* SpEX

The endotoxin concentration in purified recombinant SpEX-M was measured using a ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (Genscript). Recombinant SpEX-M at 20 µg was diluted in 0.5 cc in phosphate buffered saline (PBS) (pH 7.2) and injected into three clinically normal dogs subcutaneously in the lateral thorax. Three injections were given 7 days apart with a control dog receiving PBS (pH 7.2) only. Blood was drawn on days -7, 8, 15 and 29 (relative to injections) and serum separated.

Enzyme-linked immunosorbent assay

Recombinant SpEX and SpEX-M were coated separately onto ELISA plates (Corning) at 2µg/ml in PBS. The plates were washed with 0.05% PBS-T and incubated with two-fold serial dilutions of serum from dogs (injected with recombinant proteins) for 1 h at 37°C, then bound IgG was detected using HRP-conjugated goat anti-dog IgG-heavy

and light chain (Bethyl Laboratories, Inc.). ELISA assay plates were washed three times with PBS-T between all incubations, bound antibodies were detected using TMB substrate (Thermo Scientific), and reactions were stopped with 0.18 M sulphuric acid and optical density read at 450 nm on a plate reader (Bio TEK, EL800). The experiment was repeated a minimum of three times and a p-value of <0.05 was considered significant for all the experiments unless otherwise stated.

Trans-well assay for neutrophil transmigration

Canine blood was collected from healthy dogs using a sterile blood collection system with EDTA anticoagulant (BD Vacutainer). Canine neutrophils were isolated according to O``Donnell (158), washed several times, and re-suspended in pre-warmed 1ml RPMI medium.

A neutrophil transmigration assay protocol was used as previously described (159) with modifications. The assay was performed in 24-well plates with polycarbonate membrane (3.0 µM pore size and 6.5 mm well diameter) chambers (Corning Incorporated). Six hundred microliters of Dulbecco's Modified Eagle Medium (DMEM) with 10% of fetal bovine serum, as a PMN chemoattractant, was placed in each bottom well. Subsequently, SpEX and SpEX-M treated canine neutrophils (1×10^6), were added to the top chamber and incubated at room temperature for 6 h. DMEM medium was used as a negative control. Neutrophil viability and migration to the bottom chamber was quantified using a Countess ^{TM} II FL Automated Cell Counter (Thermo Scientific). In order to measure the protective effect of anti-SpEX on neutrophil transmigration, recombinant SpEX was incubated with canine anti-SpEX at a dilution of 1:100 for 30 min at 37°C, then tested with the PMN transmigration assay.

Complement C5 binding assay

Human complement component C5 (Sigma-Aldrich) was coated onto ELISA plates (Corning) at 2µg/ml in PBS. Recombinant SpEX and SpEX-M were added at 0.5, 1, 2 and 4 µg/ml in PBS (pH 7.2) for 1 h at 37°C, then bound recombinant proteins were detected using (HRP)-conjugated anti-6xhis tag monoclonal antibody (Thermo Scientific) at a dilution of 1:1000 in PBS-T (pH 7.2). Bound antibodies were detected as described above. The experiment was repeated a minimum of three times and a p-

value of <0.05 was considered significant for all the experiments unless otherwise stated.

Complement mediated hemolysis assay

A hemolysis assay was performed to determine the ability of recombinant SpEX to inhibit complement function. Bovine erythrocytes, collected in EDTA from clinically normal cattle, were washed in PBS and sensitized to complement by incubation with rabbit IgG fraction anti-bovine red blood cells (ICN Cappel) diluted 1:25, for 30 min at 37°C with gentle mixing. Dog serum diluted 1:4 was pre-incubated with 0.5, 1, 2 and 4 µg/ml of recombinant native SpEX for 30 min at 37°C with gentle shaking (100 rpm). One hundred microliters of sensitized bovine RBCs were added to all samples including positive and negative controls and incubated under the same conditions for an additional 30 min. After centrifugation at 4200 ×*g* for 5 min, the absorbance of the supernatant was measured at 450 nm. Heat-inactivated serum mixed with PBS (pH 7.2) was used as a negative control and normal dog serum, diluted 1:4 was used as a positive control.

PMN and monocyte permeability assay

PMNs and monocytes were separated as previously described. PMNs and monocytes were incubated with two- fold serial dilutions of recombinant proteins (SpEX or SpEX-M) from concentrations of 50 µg to 1.6 µg in a volume of 500 µl of RPMI medium supplemented with 10% fetal bovine serum in an incubator with 5% CO_2 for 30 min. The supernatant of *S. pseudintermedius* strain 06-3228 was harvested at log phase to test the toxic effect of secreted, native SpEX. PMNs and monocytes were stained with 1 µl Sytox green (Life Technologies, Inc.) for 30 min, then washed twice with PBS (pH 7.2) and analyzed using a flow cytometer (Attune acoustic focusing cytometer) by gating separately on PMNs and monocytes.

To evaluate the protective effect of canine anti-SpEX-M on PMNs and monocytes, recombinant *S. pseudintermedius* SpEX, at concentration of 3.1 µg diluted in 1ml of PBS (pH 7.2) was incubated for 30 minutes at 37ºC with serum from SpEX-M injected dogs. The cell death cut-off used for flow cytometry analysis was established using

leukocytes incubated without SpEX. Mean fluorescent intensity was measured from all gated cells.

Statistical analysis

Each experiment was repeated at least three times with a minimum of duplicate samples. Data analysis was conducted for each response variable using ANOVA methods, with treatment and dose as the independent variables. Diagnostic analysis was performed to check the model assumptions for normality and equal variance. Multiple comparisons were made with tukey's adjust. Significance was identified at $p<0.05$. All analysis was conducted in SAS 9.4 for Windows x64 from SAS Institute (Cary, NC) and some graphical outputs were generated by GraphPad Prism software (Version 7, GraphPad Software Inc.).

Acknowledgements

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CHAPTER 6
IDENTIFICATION, CLONING AND CHARACTERIZATION OF 5′-NUCLEOTIDASE PRODUCED BY *STAPHYLOCOCCUS PSEUDINTERMEDIUS*

A version of this chapter was submitted to a scientific Journal for publication. No revisions were made to the original published manuscript beyond general formatting to allow consistency through the dissertation. My contributions to this paper include: (i) selection of isolates (ii) experimental design and set up (iii) experimental testing (iv) data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) writing.

Abstract

S. pseudintermedius is a major opportunistic bacterial pathogen and the leading cause of pyoderma in dog. It is often associated with infections of the urinary system, wound and sometimes infects people. Widespread antimicrobial resistance has made the development of alternative treatments a high priority. The development of a staphylococcal vaccines, however, has proven challenging. Identification of virulence factors that inhibit phagocytosis and avoid innate immunity may play a significant role in preventing or treating infection with *S. pseudintermedius*. In this study, we identified a putative 5′-nucleotidase provisionally named SpAdsA, an *S. pseudintermedius* cell- wall protein encoded by *SpAdsA*. SpAdsA shares approximately 52 % identity with the orthologous protein of *Staphylococcus aureus* and 20 % identity with that of *Streptococcus suis* type*2*. It catalyzes the dephosphorylation of adenosine mono- and triphosphates producing adenosine that suppresses phagocytosis and facilitates bacterial survival. Attenuation of this enzyme with critical amino acid substitutions nearly eliminated its hydrolytic activity. Exogenous adenosine inhibited phagocytosis of *S. pseudintermedius* by canine neutrophils and monocytes. Conversely, the addition of SpAdsA inhibitor or A_{2A} adenosine receptor antagonist impaired the capacity of *S. pseudintermedius* to escape from killing by phagocytic cells. Antibody against SpAdsA-M was developed in clinically healthy dogs and the neutralizing effect of those antibodies was evaluated. Taken together, these results suggest that SpAdsA likely plays an important role in *S. pseudintermedius* virulence and that attenuated SpAdsA may be an important candidate for inclusion in a vaccine against *S. pseudintermedius* infections.

Keywords: 5′-nucleotidase, *S. pseudintermedius*, SpAdsA, PMNs, immune evasion, virulence, vaccine

Introduction

S. pseudintermedius is an opportunistic pathogen. It causes disease when the host's immune system is compromised or an external skin barrier breach occurs, such as wounds and surgical procedures or other factors that predispose the host to atopic dermatitis. *S. pseudintermedius* is often isolated as a common inhabitant of the skin and mucosa from noses, body, forehead, groin and anal region of healthy dogs and cats (6).

S. pseudintermedius is a coagulase-positive staphylococci (CPS) with a variety of virulence factors, some of them are similar to the ones described for *S. aureus* (15) such as proteases, leucocidin (17) and protein A (16, 21). *S. pseudintermedius* can produce various toxins including leukotoxins (17) and enterotoxins that cause substantial damage to host tissue and adversely affect host defenses (16, 17).

Methicillin resistance and associated multidrug resistance to antimicrobials available to veterinarians is common among *S. pseudintermedius* and presents a treatment challenge (9). The development of alternative therapies is, therefore, a high priority.

There are no licensed *S. pseudintermedius* vaccines. This is likely due to a lack of information about *S. pseudintermedius* protein functions, surface accessibility and epitope conservation. Information about other species of bacteria, especially staphylococci, is useful in the development of a *S. pseudintermedius* vaccine, however, this species is unique with regard to its disease presentation, genetic structure and composition, host range, and the virulence proteins it produces. Whole bacterial staphylococcal vaccines have not been efficacious. With an estimated greater than 2,000 different proteins produced by each bacterium, it is essential to identify and direct the immune response against the most important antigen targets. However, many of these proteins are potentially harmful to animals and must be attenuated to make them safe without eliminating epitopes that induce a protective immune response. Effective staphylococcal defenses are in the bacteria capacity to neutralize and/or destroy key components of their host immune system.

Upon infection or cell damage, high amounts of adenosine triphosphate (ATP), adenosine monophosphate (AMP), adenosine diphosphate (ADP) are released from damaged cells, which serve as mediators of inflammation through purinergic cell surface receptor signaling (69, 70). As a protective mechanism to prevent excessive damage to host tissue, adenosine counteracts the ATP's effect by adenosine receptor stimulation to suppress the pro-inflammatory response. Four adenosine receptors have been described: A_{1R}, A_{2AR}, A_{2BR} and A_{3R}. Neutrophils express all four adenosine receptors (71, 72). They are the most abundant mammalian leukocytes and form a key component of the innate immune response against staphylococcal infection (73, 74).

In mammals, extracellular nucleotide and adenosine concentrations are controlled by nucleotide metabolizing enzymes. In this two-step process, CD39 catalyzes dephosphorylation of ATP and ADP to produce AMP. 5′-nucleotidase (CD73) contains a characteristic two-signature sequence domain and hydrolyzes AMP into adenosine (74, 75). Some bacilli employ adenosine synthase to interfere with the balance between extracellular nucleotides and adenosine by perturbing the adenosine level beyond the physiological range (76, 77). Extracellular 5′-nucleotidases have been identified in *Staphylococcus aureus* and *Streptococcus suis* type 2 (75, 78) and shown to play an important role in evasion of the innate immune response.

Thammavongsa et al, (75) demonstrated that *S. aureus* may avoid phagocytic clearance in blood and identified 5′-nucleotidase (also named adenosine synthase A (AdsA)), a cell wall–anchored enzyme that converts adenosine monophosphate to adenosine, as a major virulence factor (72, 75). *S. aureus* AdsA has two signature sequences, the first nucleotidase signature sequence contain residues essential for divalent metal cation binding (75), whereas the second signature sequence is responsible for the hydrolytic activities of AdsA (75).

In this study, we identified a novel cell wall-anchored 5′-nucleotidase in *S. pseudintermedius* (SpAdsA) that has no more than 52 % identity with other bacterial adenosine synthase genes, but which contains conserved functional domains.

Adenosine synthase activity has not been previously described in *S. pseudintermedius* or any members of the *Staphylococcus intermedius* group. The specific objectives of this study were to characterize the immunobiological properties of *S. pseudintermedius* SpAdsA and attenuated (reduced toxicity) SpAdsA (SpAdsA -M) by measuring their inhibitory effects on phagocytosis. Antibody against SpEX-M was developed in clinically healthy dogs and the neutralizing effect of those antibodies was evaluated.

Results

Bioinformatics Analysis and *S. pseudintermedius* SpAdsA Characteristics

S. pseudintermedius SpAdsA showed significant homologies to members of the 5′-nucleotidase/apyrase protein family (Interpro accession number: IPRO006179) with two characteristic domains. Dimetal-containing phosphoesterases (DMPs) (Interpro

accession number: IPRO29052) are between position 147 and 401 with two domain signature sequences D1 (ILHTNDIHGRM) and D2 (YDAMTAGNHEFD) **(Figure 6.1)** and a 5′-nucleotidase C-terminal domain (Interpro accession number: IPRO008334) between positions 447 and 599 **(Figure 6.1)**. Furthermore, the protein sequence contains a predicted N-terminal signal peptide sequence from positions 1 through 24 and a LPXTG cell wall anchor sequence between positions 748 and 752 **(Figure 6.1)**.

Only limited identity of sequence was found with *S. aureus* AdsA (52.4%), *Enterococcus fecalis* 5′-nucleotidase (36.0%), *S. pyogenes* S5nA (20.1%) and S*. suis* Ssads (20.0%) (**Figure 6.2**). No other species of bacteria with homologous proteins were identified in the database.

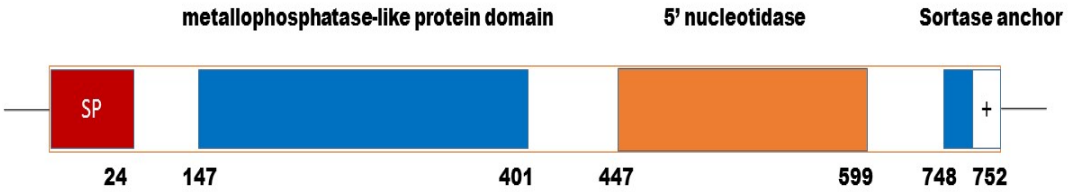

Figure 6.1 schematic presentation of SpAdsA of *S. pseudintermedius*

A BLAST search of the GenBank database revealed related putative nucleotidases in other pathogenic bacterial species within the *Staphylococcus intermedius* group including *Staphylococcus delphini* (90.8% amino acid identity), *Staphylococcus intermedius* (88.5% amino acid identity) and *Staphylococcus cornubiensis* (87.8% amino acid identity).

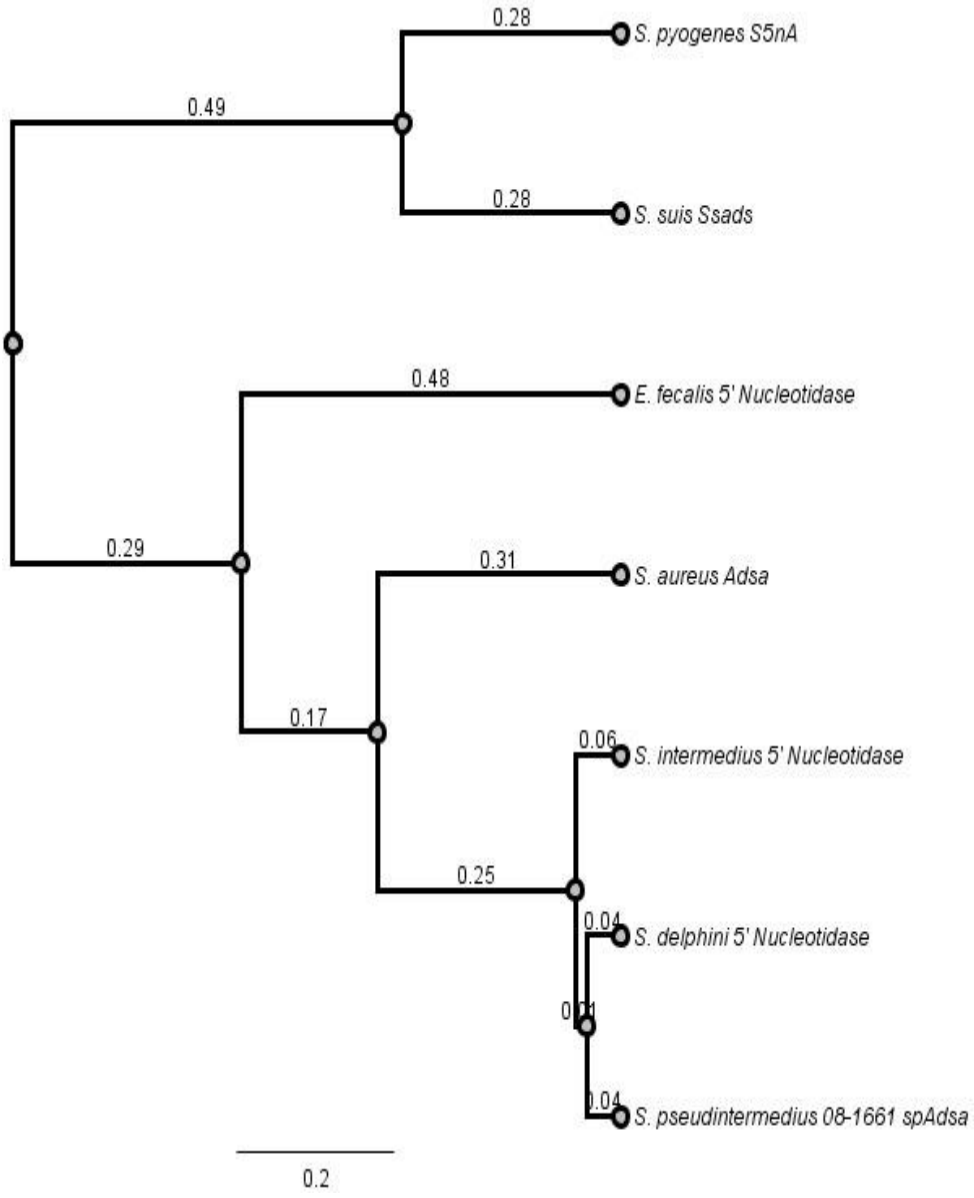

Figure 6.2 Phylogenetic tree of 5′-nucleotidase

The rooted phylogenetic tree (unweighted pair group method with arithmetic mean) was generated with Geneious version 9.1.3 using complete 5′-nucleotidase protein sequences from each bacterial species. The gap open penalty was set on 12 and BLOSUM62 was used as the cost matrix.

Multiple sequence alignment (MSA) analysis showed that SpAdsA is conserved among *S. pseudintermedius* strains including 06-3221, 08-1661and NA45 that represent the major sequence types occurring in the United States as determined by multilocus sequence typing (82). The 5′-nucleotidase gene contains an open reading frame in *S. pseudintermedius* that is 2,343 bp in length with amino acid identities over 99.4 % between strains.

Bioinformatics analysis of *S. pseudintermedius* SpAdsA showed that aspartic acids (D) (positions 154 and189), histidine (H) (positions 156, 318, 354 and 356) and asparagine (position 221) within the SpAdsA signature sequences are essential for divalent metal cation binding. Based on the previously reported Gram-positive bacterial nucleotidases and our 3D model we designed mutant protein SpAdsA (SpAdsA-M) where aspartate (position 154 and 225) and histidine (position 156 and 222) were substituted at these positions in *S. pseudintermedius* 08-1661 SpAdsA referenced to Genbank accession No: ANS88668.1 (**Figure 6.3**).

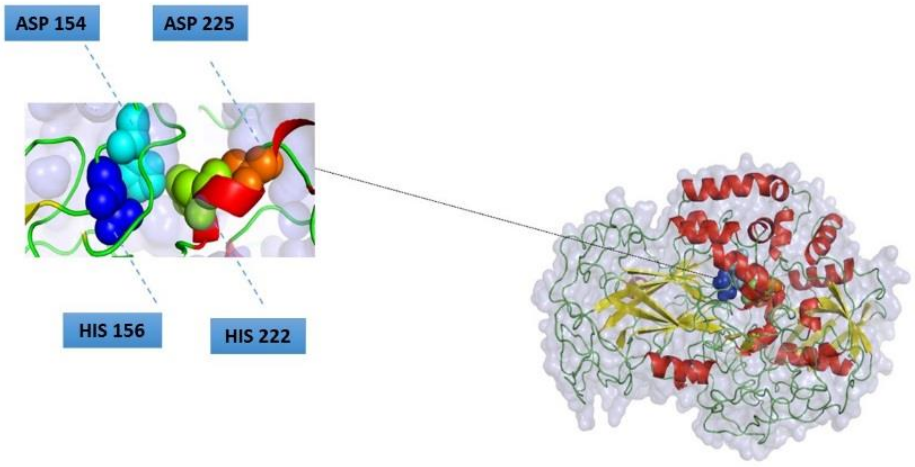

Figure 6.3 three-dimensional model of *S. pseudintermedius* SpAdsA protein

The critical amino acids ASP at positions 154 (cyan), HIS at positions 156 (blue), HIS at positions 222 (light green), ASP at positions 225 (orange) within the SpAdsA signature sequences D1 (ILHTN**D**I**H**GRM) and D2 (YDAMTAGN**H**EF**D**) were substituted with alanine to produce the mutant protein.

Cloning, expression and purification of recombinant *S. pseudintermedius* SpAdsA

SpAdsA is predicted to have an N-terminal signal sequence and C-terminal sortase anchoring domain. Recombinant SpAdsA proteins lacking those sites were produced to facilitate a study of the mature protein. The sizes of recombinant native and mutant SpAdsA were 55.8 kDa and 55.4 kDa respectively, as determined by western blot using monoclonal antibody directed against the tag. SpAdsA -M expressed well from E. coli and remained soluble in solution, indicating protein stability with the amino acid substitutions **(Figure 6.4)**.

S. pseudintermedius 5′-nucleotidase synthesizes adenosine

Tryptic digests of surface proteins of *S. pseudintermedius* 06-3228 showed hydrolytic activity that released adenosine from AMP and ATP **(Figure 6.5)**. Similar activity was obtained with recombinant SpAdsA which harbors a domain with the two signature sequences, ILHTNDIHGRM and YDAMTAGNHEFD. The total release of inorganic phosphate (Pi) by recombinant SpAdsA (mean Pi moles phosphate in 50 µl = 7998.32) as well as 06-3228 tryptic digest (mean Pi moles phosphate in 50 µl = 6688.42) from 50 µl of 1mM ATP and AMP was significantly higher than that from the mutant protein (mean Pi moles phosphate in 50 µl = 555.79) ($p<0.001$) (Figure 6.5). This suggests that the two signature sequences (D1 and D2) are critical for enzymatic activity in *S. pseudintermedius* like that of S. suis type 2 (78). Together these results confirm that SpAdsA is capable of hydrolyzing ATP and AMP to produce adenosine.

SpAdsA-M stimulates specific antibody responses against 5′-nucleotidase in dogs

ELISA analysis of sera obtained from dogs on days −7, 8, 15, and 29 (relative to the first injection of SpAdsA-M) demonstrated that antibodies to SpAdsA and SpAdsA -M **(Figure 6.6)** on day 15 were detected and on day 29 were found to be at the highest level ($P < 0.0001$) compared to the sera pre-injection control.

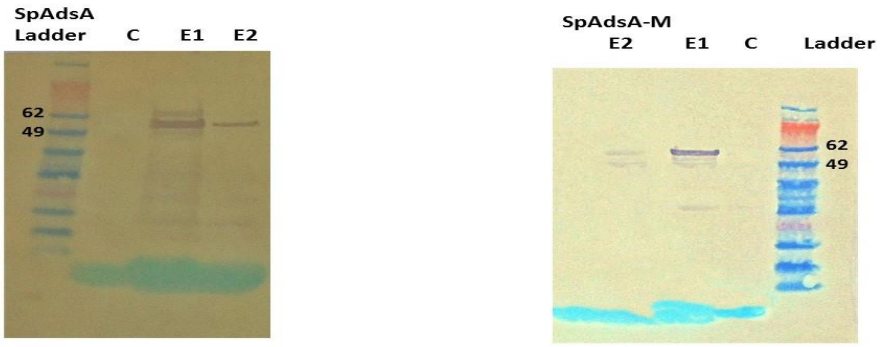

Figure 6.4 Western blot of recombinant *S. pseudintermedius* native and mutant SpAdsA detected with HRP-conjugated anti-6xhis.
The Molecular weights of recombinant SpAdsA and SpAdsA -M, after expression in Tuner ™ (DE3) pLacI *E. coli* induced by Isopropyl β-D-1-thiogalatopyranoside (IPTG), determined in western blots were of the expected sizes (55.8 and 55.4 kDa, respectively) in the elution fractions (E1 and E2). C stand for elution control.

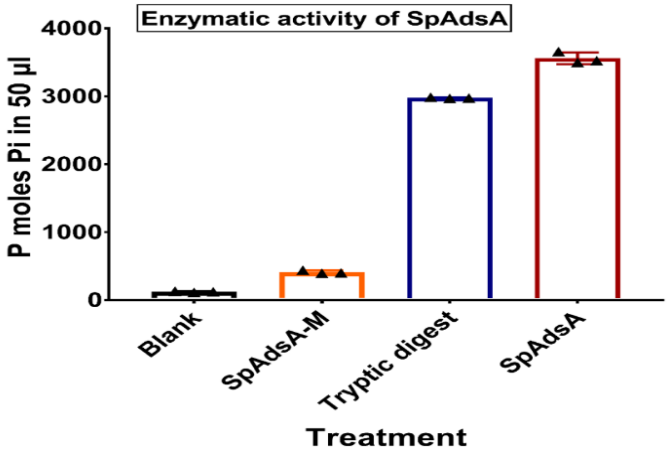

Figure 6.5 *S. pseudintermedius* 5'-nucleotidase synthesizes adenosine
The total release of inorganic phosphate (Pi) by 5' nucleotidase as well as 06-3228 tryptic digest in P moles from 50 µl of 1mM ATP and AMP was significantly higher than that from the mutant version of 5' nucleotidase. The mean IP release (+standard error) following treatment (a) and protein treatment (b). N=12 per adenosine mono- and triphosphates group and N=9 per protein treatment. Different letters above the bar indicate significant differences among treatments (p<=0.001).

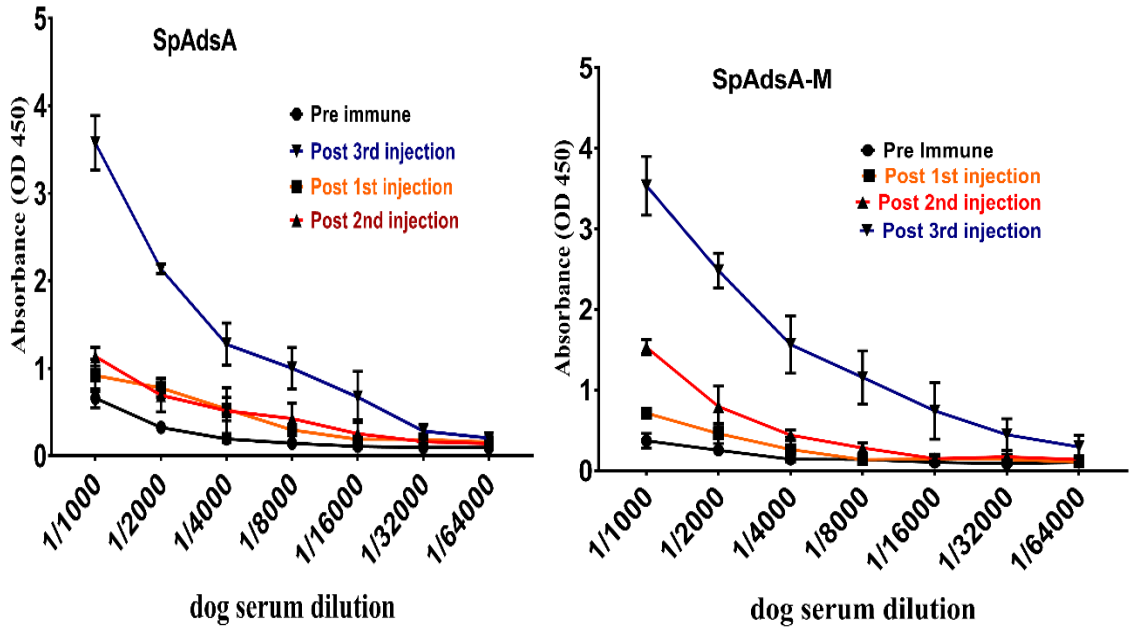

Figure 6.6 Dogs injected with SpAdsA-M developed IgG specifically reactive with recombinant native SpAdsA and SpAdsA-M

Antibodies against *S. pseudintermedius* native SpAdsA and SpAdsA-M were measured using an indirect ELISA. Recombinant *S. pseudintermedius* SpAdsA and SpAdsA-M proteins were coated on ELISA plates, then incubated with two-fold serially diluted serum from dogs vaccinated with the same proteins. High reactivity with SpAdsA and SpAdsA-M occurred with sera collected two weeks after the third injections of SpAdsA-M ($P < 0.0001$) compared to pre-injection sera. The values represent averages from three independent experiments.

SpAdsA inhibits phagocytosis of *S. pseudintermedius* in canine blood

To investigate the potential immune evasive attributes of SpAdsA, the effect of adenosine on the survival of *S. pseudintermedius* strains 06-3228 and 08-1661 in canine blood was examined. As shown (**Figure 6.7**) adenosine at a concentration of 15 µM significantly promoted *S. pseudintermedius* strains 06-3228 survival greater than 06-3228 without treatment at 60 minutes ($p<0.0001$), 90 minutes ($p<0.0001$) and at 120 minutes ($p=0.0046$). For *S. pseudintermedius* strains 08-1661 adenosine at the same concentration significantly promoted bacterial survival compared to bacteria without treatment at 60 minutes ($p<0.0001$) and 90 minutes ($p<0.0001$) and there was no significance difference at 120 minutes. Conversely, 5′-(α, β-methylene) diphosphate at concentration of 500 µM, a 5′-nucleotidase inhibitor (also called ADP analog and CD73 inhibitor), significantly diminished the ability of staphylococci to escape from phagocytic killing compared to 06-3228 without treatment at 60 minutes, 90 minutes and 120 minutes ($p<0.0001$). For 08-1661, CD73 inhibitor treatment showed significant difference at 60 minutes ($p=0.0002$), 90 minutes ($p=0.0146$) and 120 minutes ($p=0.0034$) (**Figure 6.7**). These results suggest that extracellular adenosine produced by SpAdsA inhibits phagocytic cell activity and promotes bacterial survival.

To determine the role of A_{2A} receptors on canine PMNs in the signaling pathway of adenosine we performed pharmacological inactivation of A_{2A}R using the A_{2A} receptor antagonist ZM241385. With ZM241385 (1 µM), survival of *S. pseudintermedius* 06-3228 and 08-1661was significantly decreased at all of time points (06-3228: $p<0.0001$; 08-1661: $p<0.0001$) compared to the same bacterial strains without the antagonist **(Figure 6.7).** Collectively, these results showed that adenosine has an inhibitory effect on phagocytosis, and it mediates its inhibitory signals through A_{2A} receptors on the surface of canine neutrophils.

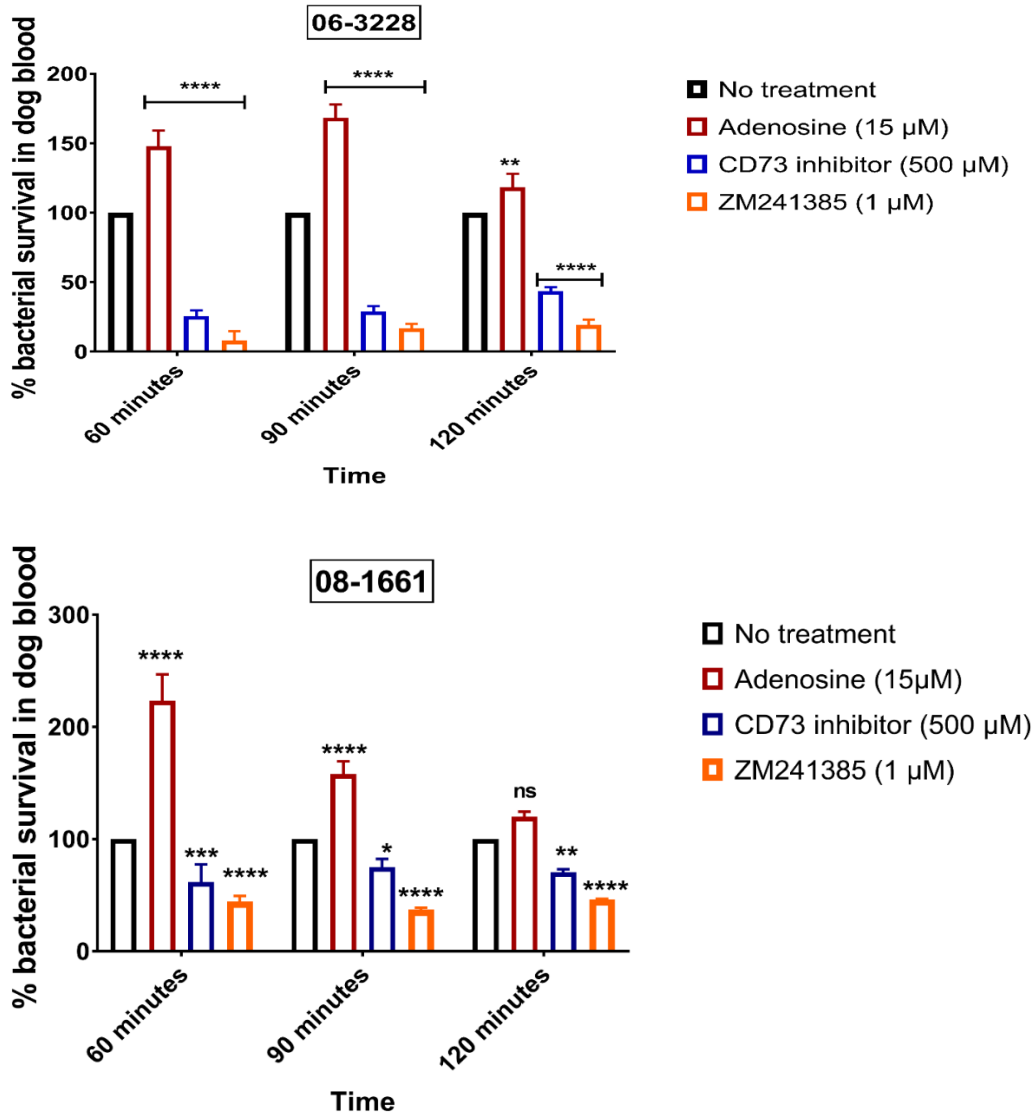

Figure 6.7 SpAdsA inhibits phagocytosis of *S. pseudintermedius* in canine blood through A2A receptors

The difference between *S. pseudintermedius* survival in canine blood from those with different treatments (exogenous Adenosine (15μM), CD73 inhibitor (500μM) and ZM241380 (1μM)) was calculated at different time points (60 minutes, 90 minutes and 120 minutes) N=3 per treatment. * Above the lines indicates a significant difference between the treatments at the corresponding time. For *S. pseudintermedius* 06-3228 ** (p=0.0046) and **** (p<0.0001). For *S. pseudintermedius* 08-1661 * (p=0.0146), ** (p=0.0034), *** (p=0.0002) and **** (p<0.0001).

Dog anti SpAdsA -M reduce the effect of SpAdsA on phagocytosis *in vitro*

Pre-incubation of *S. pseudintermedius* with dog anti SpAdsA -M at dilutions 1:10 and 1:100 in PBS and bacterial survival assay in dog blood was performed as previously mentioned. Dog anti- SpAdsA-M significantly increased phagocytosis of *S. pseudintermedius* 06-3228 than 06-3228 alone in dog blood after incubation periods of 60 minutes, 90 minutes and 120 minutes **(Figure 6.8)**.

Discussion

This study showed how *S. pseudintermedius* modulates neutrophil function by perturbing adenosine levels using SpAdsA residing on its cell wall. It demonstrates that recombinant SpAdsA and *S. pseudintermedius* cell wall proteins containing SpAdsA hydrolyze ATP and AMP generating inorganic phosphate and adenosine. A search of the GenBank database for SpAdsA protein in the other three members of the *Staphylococcus intermedius* group (SIG) found that 5′-nucleotidase produced by members of this group share over 81% amino acids identity. Furthermore, we found SpAdsA share no more than 52% amino acid identity with proteins produced by *S. aureus*, *Enterococcus* and Streptococcal species. Adenosine exerts an immunosuppressive effect through interaction with purinergic receptor 1 during infections (69, 70). Purinergic receptors are specific for extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) and are conserved among mammalian cell types (69, 70).

Neutrophils play a crucial role as professional phagocytes in the host defense against pathogens. They produce adenosine and can respond to it via G-protein coupled receptors expressed on their surface in a dose dependent manner (160). Extracellular adenosine is essential for the elimination of inflammation but it is also harmful to build up high adenosine concentrations (161, 162). Disturbance of the immune homeostasis maintained by adenosine through perturbing its level is likely to affect immune response of the host during infection. At low adenosine concentration, A_1 and A_3 receptor subtypes bound with adenosine stimulate neutrophil chemotaxis and

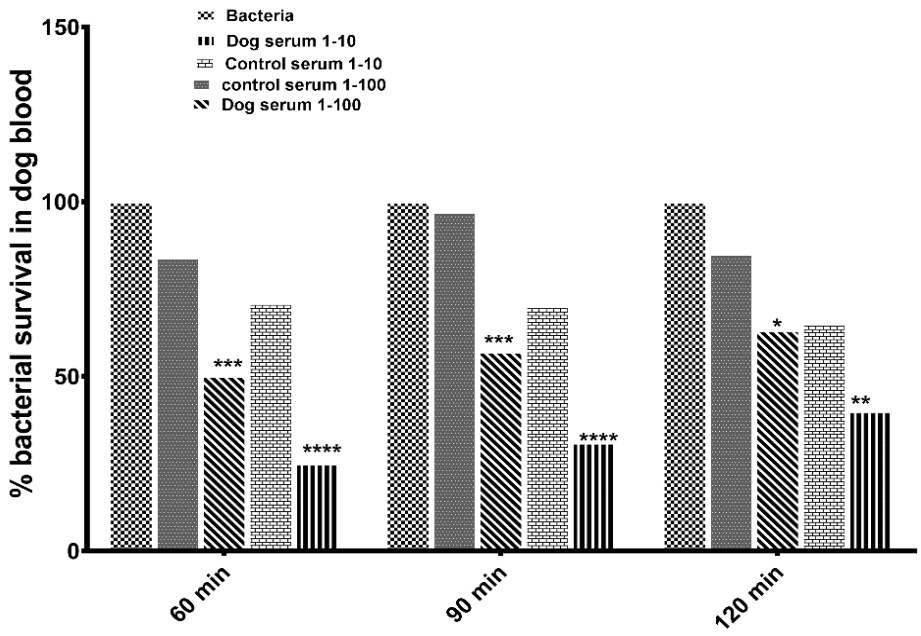

Figure 6.8 Dog anti SpAdsA -M reduce the effect of SpAdsA on phagocytosis *in vitro*

The difference between *S. pseudintermedius* survival in canine blood from those with different treatments (dog anti SpAdsA -M at dilutions 1:10 and 1:100 in PBS and control serum at the same dilutions) was calculated at different time points (60 minutes, 90 minutes and 120 minutes) N=3 per treatment. * Above the lines indicates a significant difference between the treatments at the corresponding time. * (p=0.0241), ** (p=0.0083), *** (p=0.0019) and **** (p<0.0001).

phagocytosis while at higher levels adenosine exerts its inhibitory effect through A_{2A} and A_{2B} receptors to inhibit neutrophil trafficking, oxidative burst, and release of inflammatory mediators (160). Eppell et al (76) showed that adenosine equally inhibits IgG Fc and complement-mediated phagocytosis in cultured human peripheral blood derived macrophages.

SpAdsA was considered a good candidate for producing adenosine synthase due to the presence of conserved domains associated with this enzymatic activity in other species of bacteria. Roche et al (163) showed that *S. aureus* 5' nucleotidase has a crucial role in invasion. Thammavongsa et al. (75) further distinguished a fundamental role for *S. aureus* 5' nucleotidase in disease. Previous studies demonstrated the importance of *S. suis* type 2 adenosine synthase (78) but there have not been any reports on immune evasive attributes of 5' nucleotidase produced by *S. pseudintermedius* or studies of it with canine phagocytes. Native and mutant 5' nucleotidase proteins excluding signal peptides and anchoring sequences were used for this study because the full length expressed proteins were not readily soluble in aqueous solution and would not represent mature proteins (78). Enzymatic inhibition using an ADP analogue or A_2 receptor antagonist significantly reduced bacterial survival in dog blood suggesting that *S. pseudintermedius* SpAdsA plays an important role in canine pathogen immune evasion and may serve as an important candidate target for novel anti-infective drugs and vaccines against canine pyoderma.

We used an A_2 receptor inhibitor to determine if adenosine signaling occurs through the A_2 receptor in canine cells and found that the addition of ZM241385 stimulated phagocytic cell activities against *S. pseudintermedius.* This is consistent with the finding of Jordan et al (164) although they used the selective adenosine A_2 agonist CGS-21680 that activated A_2 receptors and reduced cardiac reperfusion injury and infarction size (165-169) by inhibiting neutrophil accumulation and superoxide anion production in a canine model of ischemia and reperfusion.

The present study revealed the diminished ability of canine blood to kill *S. pseudintermedius* in the presence of exogenous adenosine. Conversely, addition of ADP analogue or ZM241385 prevented *S. pseudintermedius* escape from phagocytic killing

which is consistent with the findings of Barletta et al (160) and Jordan et al (164) studying ischemia and perfusion in a canine model using, *S.suis type2* SasH (78) and *S. aureus* AdsA (75), respectively.

These results indicate that SpAdsA plays an important role in promoting *S. pseudintermedius* survival and inhibiting PMN phagocytosis and killing activity by the synthesis of adenosine. The production of neutralizing antibody in healthy dogs suggests that attenuated, non-toxic and immunogenic recombinant protein produced in this study is a good candidate against *S. pseudintermedius* infection.

Materials and Methods

Ethics Statement. The University of Tennessee Institutional Animal Care and Use Committee (IACUC) has reviewed and approved experimental protocols, including 2474-0716 for samples of dog blood and 2572-1217 for producing antibodies in dogs with recombinant protein.

Bacterial strains and plasmids

The *S. pseudintermedius* strains, the most common sequence (ST) in the United States, were used in this study (82, 84, 114), included 06-3228 (ST68), 08-1661 (ST71) and NA45 (ST84) (82, 84, 114). Strains 06-3228 and 08-1661 were isolated at the University of Tennessee, College of Veterinary Medicine Bacteriology Laboratory. Strain NA45 was a gift of Faye Hartmann of the University Of Wisconsin School Of Veterinary Medicine.

Plasmid construct pMA-RQ-Bs- M Ado $_{\mathbf{370\text{-}1868}}$, containing a mutated synthetic *S. pseudintermedius SpAdsA* gene with BamHI/NotI cloning sites was obtained commercially (Invitrogen™ GeneArt™ Gene Synthesis - Thermo Fisher Scientific).

Bioinformatics Analysis and *S. pseudintermedius* 5′-nucleotidase characteristics

Rooted phylogenetic tree (UPGMA (unweighted pair group method with arithmetic mean)) of 5′-nucleotidases was generated with Geneious version 9.1.3 (107) using complete protein sequences of SpAdsA (*S. pseudintermedius* 08-1661, ANS88668.1), S5nA (S. pyogenes, AAK33792)(170), AdsA (*S. aureus*, ESR29110)(75), Ssads (*S. suis,* YP001197640)(78), 5′-nucleotidases (*Staphylococcus delphini,* WP_096556274.1), 5′-nucleotidases (*Enterococcus faecalis,* WP_010714156.1) and 5′-nucleotidases

(*Staphylococcus intermedius,,* WP_086428047.1). Multiple sequence alignment of *S. pseudintermedius* 5′-nucleotidase proteins was performed. The bacterial localization prediction tool, PSORTb version 3.0.2 (available at http://www.psort.org/psortb/) (108) was used to predict the topology and domain structure of SpAdsA protein. Protein modeling and binding site prediction was performed using Phyre^2 web portal (available at http://www.sbg.bio.ic.ac.uk/phyre2/) (109) and 3DLigandSite (available at http://www.sbg.bio.ic.ac.uk/3dligandsite/)(110).

Polymerase chain reaction (PCR) amplification of the wild and mutant *SpAdsA* gene from *S. pseudintermedius*

Single bacterial colony of *S. pseudintermedius* strain 08-1661 obtained from blood agar plates were grown in tryptic soy broth (TSB) (Becton, Dickinson and Co., Sparks, MD) at 37°C with 225 rpm shaking. MO BIO UltraClean® Microbial DNA Isolation Kit (QIAGEN Inc. Cat No.12224-50) was used to extract the bacterial DNA according to the manufacturer instructions. Oligonucleotide primers with BamHI and NotI restriction sites (underlined) were designed using the PrimerQuest Tool (https://www.idtdna.com/Primerquest/Home/Index) based on the complete genome sequence of *S. pseudintermedius* strain 08-1661(9).

Primers for the amplification of native *SpAdsA* were

W-Ado-Forward: GCATGAGGATCCGAAACGACTGCAACGCATAC and

W-Ado-Reverse: GCATGAGCGGCCGCTCCACCTGAAGCTGTAAAGTC.

Primers for amplification of mutant *SpAdsA* were

M-AdoForward: GCATGAGGATCCATGTTAAGATTGTCGGCTAAAAAAG and

M-AdoReverse: GCATGAGCGGCCGCTCCACCTGAAGCTGTAAAGTC.

The native SpAdsA open reading frame (ORF) (1539 bp) without the regions encoding the predicted N-terminal signal and C-terminal sortase anchoring sequence was amplified from 60 ng of genomic DNA of *S. pseudintermedius* 08-1661 by 35 cycles of PCR with rtaq polymerase (Takara, Cat No. R004) at an annealing temperature of 55 ºC using primer W-Ado-Forward and W-Ado-reverse. The ORF of mutant SpAdsA (1536bp) was excised from pMA-RQ-Bs- M Ado plasmid using BamHI- NotI restriction enzymes.

Cloning and expression of recombinant wild and mutant *SpAdsA* of *S. pseudintermedius* 08-1661

The PCR products of wild and mutant *SpAdsA* were digested with the restriction enzymes BamHI and NotI and purified using QuickClean II Plasmid Miniprep Kit (Genscript, Cat No. L00420) according to the manufacturer instructions. Purified product was ligated into pETBlue™-2 (Novagen, WI. USA), transformed into DH5-alpha *E. coli* and plated on LB agar plates with 100 µg/mL ampicillin, 80 µg/mL X-gal and 50µM IPTG (Teknova, Cat No. L1925). For screening the clones containing recombinant plasmid, X-gal chromogenic substrate was added to the agar plates. The recombinant plasmid was extracted using GeneJET Plasmid Miniprep Kit according to the manufacturer instructions (ThermoFisher, Cat No. K0502). The presence of the cloned fragment was confirmed by PCR. pETBlue™-2 constructs were transformed in Tuner ™(DE3) pLacI *E. coli* chemically-competent cells (Novagen, Cat No .70623) by heat shock and plated on LB agar containing 50µg/ml ampicillin (Sigma-Aldrich, Cat No. A5354) and 20µg/ml chloramphenicol (Sigma-Aldrich, Cat No. C0378). A single colony was used to inoculate LB broth containing 50µg/ml ampicillin and 20µg/ml chloramphenicol and the broth was incubated overnight at 37ºC with shaking at 225 rpm. Fresh LB medium containing 50µg/ml ampicillin and 20µg/ml chloramphenicol was inoculated at a 1:100 dilution and grown until an optical density (OD_{600}) of 0.4-0.6 was reached then protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalatopyranoside (IPTG) for 4 hr at 30ºC with shaking at 225 rpm.

A suspension of IPTG-induced Tuner cell bacteria (100 ml) was centrifuged at 12000 xg for 5 min. Bacteria were suspended in 5 ml of protein extraction reagent (BugBuster, Novagen Cat No. 70584) and 20 µl 100X Protease Inhibitor Cocktail Set III, EDTA-Free (Calbiochem, Cat No. 539134), incubated for 30 min at 37ºC in a shaking incubator at 225 rpm, and centrifuged at 12,000× g for 45 min at 4°C.

Recombinant protein was purified using a HisPur™ Ni-NTA Spin Purification Kit (Thermo Scientific, Cat No. 88228) according to the manufacturer instructions. Protein concentrations were determined with the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Cat No. 23225).

SDS-PAGE and Western blot

Protein samples were resolved by SDS-PAGE in 4-12 % polyacrylamide gels (Invitrogen, Cat No. NP0322BOX) and electrophoretically transferred onto a nitrocellulose membrane (Thermo Scientific, Cat No. 77010). Then the blots were incubated overnight in 5% (wt/vol) nonfat dried milk powder in 0.05% polyethylene glycol sorbitan monolaurate (Tween 20) containing phosphate buffered saline (PBS-T) at 4°C. Horseradish peroxidase (HRP)-conjugated anti-6xhis tag monoclonal antibody (Thermo Scientific, Cat No. MA1-21315-HRP) diluted 1:2,000 in 0.05% PBS-T was added to the blocked membranes and incubated for 1 h with 225 rpm shaking at room temperature. After five washes with 0.05% PBS-T, bound antibodies were detected using 1-Step™ chloronaphthol substrate solution (Thermo Scientific, Cat No. 34012).

Adenosine Synthase Activity

Overnight cultures of *S. pseudintermedius* 06-3228 and 08-1661 were centrifuged and washed twice with phosphate buffer saline (PBS) and then suspended in 1 ml digestion buffer (0.6 M sucrose buffered with 50 mM Tris, pH 7.5) (74) containing 200 µg trypsin from porcine pancreas (Sigma-Aldrich, Cat NO. T4799). Bacteria were digested with trypsin for 1.5 h at 37°C, then the supernatant containing *S. pseudintermedius* SpAdsA was obtained after centrifugation at 10,000xg for 5 minutes at 4°C. Four ml of *S. pseudintermedius* tryptic digest and recombinant SpAdsA protein at 0.15 µg/ ml were suspended in 500 µL TM buffer (50 mM Tris-HCL [pH 7.5], 5 mM $MnCl_2$), then hydrolysis of ATP (Sigma-Aldrich, Cat NO. A26209) and AMP (Sigma-Aldrich, Cat NO. 01930) was carried out in the presence of 1 mM nucleotide at 37°C for 15 minutes. Inorganic phosphate (IP) release was measured using a malachite green phosphate kit according to the manufacturer protocol (Sigma-Aldrich, Cat NO. MAK307).

Production of antibodies against recombinant *S. pseudintermedius* SpAdsA

Recombinant SpAdsA-M was injected into three clinically normal dogs at 100 µg/0.5 ml in PBS (pH 7.2) through the subcutaneous route in the lateral thorax. For a total of three injections with a control dog receiving PBS (pH 7.2) only, injections were given once every 7 days. Blood (6 ml) was collected 4 times on days −7, 8, 15 and 29 from a jugular vein

using a 20 g needle and 12 cc syringe. The collected blood was left undisturbed at room temperature for 30 min followed by centrifugation at 2,000 x g for 10 min in a refrigerated centrifuge.

Enzyme-linked immunosorbent assay

To detect a specific antibody response against SpAdsA- M in injected dogs, recombinant *S. pseudintermedius* SpAdsA and SpAdsA –M were coated separately onto ELISA plates (Corning, Cat No. 3590) at 2µg/ml in PBS (pH 7.2). The plates were washed with 0.05% PBS-T and incubated with two-fold serial diluted serum from dogs (injected with recombinant proteins) for 1 h at 37°C, then bound IgG was detected using HRP-conjugated goat anti-dog IgG heavy and light chain (Bethyl Laboratories, Inc. Cat No. A40-123-1). ELISA assays plates were washed three times with PBS-T between all incubations, bound antibodies were detected using TMB substrate (Thermo Scientific, Cat No. N301), reactions were stopped with 0.18 M sulphuric acid and optical density read at 450 nm on a plate reader (Bio TEK, EL800). The experiment was repeated a minimum of three times and a p-value of <0.05 was considered significant for all the experiments unless otherwise stated.

Bacterial survival in blood

Overnight cultures of *S. pseudintermedius* 06-3228 and 08-1661 were diluted 1:100 into fresh TSB and grown for 3 hours at 37°C. Staphylococci were centrifuged 9000 x g, washed twice, and diluted in PBS to yield an optical density (OD_{600}) of 0.5. Then 100 µl of 4×10^6 CFU per ml of bacteria were mixed with 500 µl of fresh canine blood, collected in EDTA. The samples were incubated at 37°C with 100 rpm shaking and harvested at 60, 90 and 120 minutes. They were incubated on ice for 30 minutes in 1 ml of Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich, Cat NO. R7757), then bacteria were pelleted at 9,000 x g at 4°C and plated on tryptic soy agar (TSA) for enumeration of viable bacteria. To determine the effect of canine anti SpAdsA- M on phagocytic cells, *S. pseudintermedius* was incubated for 30 minutes at 37ºC with serum from SpAdsA- M injected dogs at two different dilutions 1:10 and 1:100 in PBS (pH 7.2) with serum from

saline injected dogs at the same dilutions was used as a control. The experiment was run in duplicate and a p-value of < 0.05 was considered significant.

Statistical Analysis

Data were analyzed using general liner modes with treatment, protein and time as the independent variables while inorganic phosphate (IP) release, mean fluorescence intensity (MFI) and colony forming units per ml (CFU ml^{-1}) were used as response variables, respectively. Multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at the significant level of 0.05. Rank transformation on response variables were conducted when the normality assumption was violated. All analyses were conducted using PROC mixed in the SAS system for Windows version 9.4 (SAS Institute Inc., Cary, NC).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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CHAPTER 7
CONCLUSIONS AND RECOMMENDATIONS

The increased incidence of methicillin resistance and multidrug resistance were connected to the *S. pseudintermedius*. Given the few antibiotics available for therapy in the veterinary world, new strategies for treating or preventing infection are essential. Vaccine growth requires understanding the essence of immune reaction against *S. pseudintermedius* infection.

Multiple *S. aureus* studies have shown that a successful vaccine can be one that can boost both B-cells and T-cells with several distinct antigenic determinants. Given these variables, mass spectrometry was evaluated for the secretome, surface-associated proteins and IgG proteins from three kinds of representative sequences. From the information, a key *S. pseudintermedius* virulence proteins were identified and characterized.

Characterization of *S. pseudintermedius* SpsQ has provided information about the immune evasive role of this protein and it is superantigenic effect on canine B cells. The amino acids in the *S. pseudintermedius* SpsQ that are critical for Ig Fc and Fab regions were identified, and a non-toxigenic form of the protein was produced and tested both *in vitro* and *in vivo* as a potential vaccine candidate.

We described a unique pore-forming toxin from *S. pseudintermedius*.

The killing effect of recombinant *S. pseudintermedius* LukS-I and LukF-I on canine polymorph nuclear leukocytes was determined. The cytotoxic effect occurred only when the two recombinant proteins were combined. Engineered mutant versions of the two-component pore-forming leucocidins, produced through amino acids substitutions at selected points, were not cytotoxic. Anti-Luk-I produced in dogs against attenuated proteins reduced the cytotoxic effect of native *S. pseudintermedius* leukotoxin, which highlights the importance of Luk-I as a promising component in a vaccine against canine *S. pseudintermedius* infections.

S. pseudintermedius exotoxin provisionally named SpEX was identified. This exoprotein binds to complement component C5, prevents complement mediated lysis of sensitized bovine red blood cells, kills polymorphonuclear leukocytes and monocytes and inhibits neutrophil migration at sub-lethal concentrations. A mutant version of SpEX, produced through amino acid substitution at selected positions, had diminished cytotoxicity. Anti-

SpEX produced in dogs reduced the inhibitory effect of native SpEX on canine neutrophil migration and protected immune cells from the toxic effects of the native recombinant protein.

We identified a putative 5′-nucleotidase provisionally named SpAdsA, an *S. pseudintermedius* cell- wall protein encoded by *SpAdsA*. SpAdsA catalyze the dephosphorylation of adenosine mono- and triphosphates producing adenosine that suppresses phagocytosis and facilitates bacterial survival. Attenuation of this enzyme with critical amino acid substitutions nearly eliminated its hydrolytic activity. Antibody against SpAdsA-M was developed in clinically healthy dogs and the neutralizing effect of those antibodies was evaluated.

These results suggest that attenuated *S. pseudintermedius* proteins may be an important candidate for inclusion in a vaccine composite against *S. pseudintermedius* infections. This work characterized the critical domains and the essential amino acids sites within a key of *S. pseudintermedius* virulence proteins and that can utilize to design a chimeric protein consisting of a cocktail of functional and nontoxic domains from the identified *S. pseudintermedius* virulence proteins.

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VITA

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