DEVELOPMENT OF A CHOLERA TOXIN CTA_2/B BASED *STAPHYLOCOCCUS AUREUS* VACCINE TO PREVENT BOVINE MASTITIS

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The following individuals read and discussed the dissertation submitted by student Neha Misra, and they evaluated her presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

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DEDICATION

I dedicate this dissertation to my parents, who have been my inspiration. To my little sister, who never stopped encouraging me and to my husband for his endless support and love.

" ***The important thing is not to stop questioning. Curiosity has its own reason for existing.***"

Albert Einstein (1879-1955)

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ABSTRACT

Staphylococcus aureus is an important pathogen causing chronic and invasive disease worldwide. This bacterium is a leading cause of community and hospital acquired infections in humans, and is also known to infect wild and domestic animals. Bovine mastitis, or inflammation of the udder, is one of the most economically relevant diseases of the dairy industry, with a high incidence worldwide. *S. aureus* is a major etiological agent causing this disease. *S. aureus* mastitis is highly contagious and difficult or impossible to treat. Management practices at dairy farms, that include good sanitation and antibiotic use, have been partially successful in reducing the occurrence of this disease, however, a complete prevention or elimination is still to be achieved. Despite efforts over more than two decades, an effective vaccine for *S. aureus* mastitis, that can protect against heterologous strains of this bacterium, is not yet available. These efforts however, have improved our understanding of the pathogenicity, virulence factor expression and immune responses to this bacterium. Studies have indicated that there is significant intraspecies variability, and an effective vaccine against *S. aureus* will require the incorporation of multiple conserved and relevant antigens. Additionally, the route of vaccine administration and use of adjuvants to aid in antigen delivery and enhancement of immune responses will also be critical.

S. aureus contains a broad array of virulence factors required for colonization and disease, including: adhesins, toxins, a polysaccharide capsule, enzymes and immune evasion molecules, that are required for adhesion, invasion and colonization. Some

virulence factors are highly conserved and centrally important for bacterial survival and sustenance, making them good vaccine targets. The iron-regulated surface determinant A (IsdA) and clumping factor A (ClfA) are two such conserved *S. aureus* extracellular-matrix adhesins that are promising vaccine antigens. However, an effective *S. aureus* vaccine for the prevention or reduction of mastitis will need to induce mucosal and systemic, as well as cellular and humoral, immune responses to these antigens. Bacterial enterotoxins are well characterized vaccine adjuvants that act by enhancing the mucosal delivery of antigens and promoting both systemic and mucosal humoral responses. Cholera toxin (CT), from *Vibrio cholerae* is a gold-standard vaccine adjuvant that can promote both humoral and cellular immune responses to co-administered antigens when delivered to mucosal surfaces.

The work presented here is based upon the overarching hypothesis that *a mucosal, enterotoxin-based vaccine containing multiple relevant antigens will protect cows against S. aureus mastitis*. To construct this vaccine, three immediate aims were developed. First, to ensure the incorporation of relevant antigens, the variability, genetic conservation and immunogenicity of the IsdA protein during bovine infection was determined. Second, a cholera toxin adjuvant based vaccine containing IsdA and a second antigen, ClfA (IsdA-CTA_2/B +ClfA-CTA_2/B) was used to vaccinate cows to determine immunogenicity. Lastly, a new immunoproteomics approach was used to identify immunogenic antigens for future incorporation into a multivalent vaccine. The results from these studies, as presented in chapters 2-4, indicate that: *1)* the IsdA adhesin is expressed and conserved in bovine strains of *S. aureus*, *2)* IsdA-CTA_2/B + ClfA-CTA_2/B can stimulate significant immune responses in vaccinated animals after intranasal

administration and *3)* immunoproteomics using milk antibodies can be used to identify new potential *S. aureus* vaccine antigens. The studies presented here will contribute to the advancement and understanding of staphylococcal vaccines in general, and specifically to those that will prevent bovine mastitis.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cells
CFTR	Cystic fibrosis transmembrane receptor
CT	Cholera toxin
CTB	Cholera toxin B subunit
CP5	Capsular polysaccharide 5
CP8	Capsular polysaccharide 8
ClfA	Clumping factor A
ClfB	Clumping factor B
CDC	Center for Disease Control and Prevention
DC	Dendritic cells
EFB	Extracellular fibronectin binding protein
EsxA	ESAT-6 secretion system extracellular protein A
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	ER associated degradation
FUR	Ferric iron regulator
FG	Fibronectin
FN	Fibrinogen
FhuD2	Ferrichrome-binding periplasmic protein
GC	Germinal centers

IsdA	Iron regulated surface determinant protein A
IsdB	Iron regulated surface determinant protein B
IsdC	Iron regulated surface determinant protein B
Ig	Immunoglobulin or antibodies
IL-1β	Interleukin one beta
IL-4	Interleukin four
LIM	Low iron media
NK	Natural killer cells
NEAT	Near iron transporter
NF-κB	Nuclear Factor Kappa-B
OPA	Opsonophagocytic assays
OD	Optical density
PCR	Polymerase chain reaction
PKA	Protein kinase A
qRT-PCR	Quantitative real time PCR
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
SCC	Somatic cell count
SCID	Severe Combined immunodeficiency
TNF-α	Tumor Necrosis Factor.
TMB	Tetramethylbenzidine
2DE	Two-dimensional electrophoresis

CHAPTER ONE: CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* INFECTION AND ITS PREVENTION BY VACCINE

Background and Introduction

Staphylococcus Aureus "the super bug"

Staphylococcus aureus (S. aureus) is a gram positive pathogen that is known to colonize almost 30% of the human population, according to the CDC [1]. *S. aureus* causes a wide range of clinical disease and life-threatening conditions like pneumonia, endocarditis, toxic shock syndrome and sepsis [2]. This pathogen has long been considered a high risk to public health; causing both hospital acquired and community infections in humans. *S. aureus* is the fourth most common hospital-acquired pathogen and the second most common pathogen isolated from surgical sites, causing 9% of nosocomial infections in United States alone [3,4]. Antibiotic therapy to treat this pathogen is either ineffective or challenging due to the prominence of antibiotic resistant strains like MRSA (methicillin-resistant *S. aureus*) and VRSA (vancomycin-resistant *S. aureus),* which are resistant to almost all common use antibiotics [5]. It is estimated that more than 2 million humans are sickened every year with antibiotic resistant strains of *S. aureus* [6]. In 2011, the CDC identified 80,461 cases of severe MRSA infection, which resulted in 11,285 deaths [7].

In addition to being an important pathogen for humans, *S. aureus* colonizes and infects almost all animal species. *S. aureus* has been reported to cause severe pathologies

in pets, wildlife and livestock [8,9]. Importantly, *S. aureus* is known to cause one of the most prevalent diseases of the dairy industry, called bovine mastitis. Bovine mastitis is common in all countries worldwide, and is reported to occur in anywhere from 5% to 70% of cows, depending upon herd and location, and in up to 90% of herds [10]. Multiple pathogens can cause bovine mastitis; however, *S. aureus* caused mastitic infections are persistent, contagious, and difficult to treat, with low cure rates [11]. *S. aureus* mastitis often becomes a chronic and clinical or subclinical inflammatory disease of the cow udder caused by intra-mammary infection [12]. This is one of most economically relevant diseases of the dairy industry, causing large economic losses every year, mainly due to reduced milk yield and quality. While it is difficult to estimate the losses caused by *S. aureus* alone, the economic losses caused by bovine mastitis in general are estimated to be approximately 1.5 billion/year in US, or $100-200/cow [13–15]. Improved health management practices at dairy farms have decreased levels of *S. aureus* infection and disease, however eradication is currently far from achieved.

Attempts to design a vaccine for *S. aureus* have occurred over decades, however, an effective vaccine to prevent disease and transmission is still not available for humans or bovines. Current *S. aureus* vaccines are either inconsistent or not protective. This is likely due to the significant intra-species variability of *S. aureus* and its multitude of potent virulence factors. The pathogenesis of *S. aureus* is mediated by this array of factors, that includes; cell wall proteins, secreted factors, immune evasion molecules and toxins. These multifunctional proteins and molecules are very important for *S. aureus* proliferation and survival inside the host. Many virulence factors play a critical role in

adhesion, invasion and pathogenesis and hence their presence is highly conserved in different strains and serotypes.

A vaccine strategy that incorporates conserved virulence factors or cell wall anchored proteins as antigens will more effectively stimulate cross-protective immune responses and aid in the eradication of *S. aureus* disease and colonization. The following studies focused on developing a cholera toxin adjuvant based vaccine for *S. aureus* caused bovine mastitis. Additionally, the identification and characterization of various virulence factors involved in the infective stage of disease pathogenesis for use as vaccine targets is described in this work.

Virulence and Pathogenicity of *S. aureus*

Colonization of *S. aureus* is a risk factor for invasive disease. Skin and mucosal surfaces, like the nose, throat, vaginal wall, and gastrointestinal (G.I.) tract, are the common sites of *S. aureus* colonization [16]. The squamous epithelium of anterior nares is transiently colonized in 60%, and permanently in 20%, of the population [17]. One of the major concerns about nasal colonization is the potential dissemination of the bacterium to other parts of the body. It has been reported that when nasally colonized individuals were topically treated for their colonization in the nose, the infection rate of *S. aureus* in these individuals to other parts of body was significantly reduced [17].

The *S. aureus* surface and secreted proteins contribute to an extraordinary arsenal of virulence factors, promoting survival in extreme conditions within host [10]. These proteins, that promote colonization to host extracellular matrix (ECM), growth in serum or tissue, and avoidance of the immune system are important as vaccine antigenic targets [18]. The majority of these are either secreted from the bacterial cell or anchored to the

cell wall through sortase action. The expression of these proteins is carefully controlled by *S. aureus* to be expressed in response to the challenges encountered in the host for colonization and survival [19]. Expression is controlled by growth phases, like stationary or exponential growth, and nutritional conditions, like the limited availability of iron and other nutrients [20]. Thus the surface of the bacterium plays a critical role in interactions with the environment and the host [21]. It has been reported that *S. aureus* can express up to 24 different cell wall anchored proteins at a given time [22].

Although colonization of *S. aureus* is associated with increased risk for infection; the carriers of *S. aureus* usually exhibit less severe symptoms of disease than typically seen in non-carriers [17]. This can be explained by the presence of a higher level of anti-staphylococcal antibodies in the *S. aureus* carriers as compared to non-carriers [23]. Carriers have been found to predominantly induce antibodies to adhesive and ECM-binding proteins [24]. Adhesion to the host is a pivotal step in the pathogenesis of *S. aureus*, and is a prerequisite for colonization and infection of the host. The wide variety of proteins and factors that aid in this process are called adhesins. Bacterial adhesins mediate attachment to ECM, epithelial cells, plasma proteins, endothelial cells and other microorganisms [25]. Adhesins can be bound on the surface or can be secreted. The *S. aureus* Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) are the most prevalent group of surface associated adhesive proteins. Whereas, secreted *S. aureus* adhesins are commonly referred to as secretable repertoire adhesive molecules (SERAMs) [26]. MSCRAMMs and SERAMs bind to abundant ECM molecules like fibronectin (FN), fibrinogen (FG), vitronectin, hemin, bone sialoprotein, transferrin and collagen [27–34]. Adhesion to mucosal surfaces and

resistance to innate immunity at this surface is key to pathogenesis, and *S. aureus* is a common skin and nasal commensal. Surprisingly, animal *S. aureus* strains possess very similar adhesive and immune evasion proteins to human strains, although the targets are substantially different. Table 1-1 shows *S. aureus* adhesins and surface proteins that have been prominently important in causing infection, invasion and colonization in both the animal and human host [35].

Table 1-1 *S. aureus* virulence factors involved in adhesion to the host.

Name	Gene	Function
Fibronectin binding proteins A, B	*fnbpA, B*	Adhesins to fibronectin (FN), fibrinogen (FG) and elastin
Collagen-binding adhesin	*cnA*	Adhesins to collagen (I and IV)
Iron-regulated surface protein A (IsdA)	*isdA*	Iron sequestration, cellular hydrophobicity, resistance to lactoferrin, binds to FN, FG, haem, hemin, transferrin and fetuin.
Iron-regulated surface protein B (IsdB)	*isdB*	Binds to haemoglobin and haem, Iron sequestration
Iron-regulated surface protein C (IsdC)	*isdC*	Binds hemin, haem transport to cytoplasm
Iron-regulated surface protein H (IsdH)	*isdH*	Binds hepta-haemoglobin, iron sequestration
Serin-aspartate repeat proteins C, D and E	*sdrs*	Adhesins
Bone sialoprotein-binding protein	*bpB*	Binds bone sialoprotein and FG
Staphylococcal protein A	*SpA*	Binds Fc domain of Ig, complement factor C3, Causes degradation of immunoglobulins
Extracellular adherence protein	*eap/map*	MHC-II analog, binds to FN, FG, vitronectin and collagen, adhesion to host cells, biofilm formation
Alpha toxin	*hlA*	Pore forming toxin
Transglucosylase IsaA	*isaA*	Lytic transglucosylase of *S. aureus*
ESAT-6 secretion system extracellular protein A	*esxA*	Involved in abscess formation, virulence factor and pathogenesis
Fe (Ferrous iron transport protein B)	*feoB*	ferrous iron transmembrane transporter activity
Staphylococcal super antigen-like 5, 11,1 and 7	*Ssl 5, 11,10,7*	Neutrophil migration, binds to chemokine receptor, Fc region of IgA.
Capsular polysaccharides	*CPSs*	Alters C3 (CPS5 and 8) or C3b deposition
Clumping factor, A	*clfA*	Adhesion to FG, binds complement factor I
Clumping factor, B	*clfB*	Binds to FG and cytokeratin, skin and nares colonization

Cell wall anchored *S. aureus* proteins possess secretory or sec signal sequence on the N-terminus and sortase or sorting signal at C-terminus. The sorting signal is comprised of the LPXTG (Leu-Pro-X-Thr-Gly; X= any amino acid) motif [36]. Cell wall anchored MSCRAMMs can perform multiple functions, are structurally similar and have a common mechanism of ligand binding [37]. MSCRAMMs are often the first proteins to interact with the host, and hence support colonization and immune evasion. *S. aureus* possess much genetic plasticity and is involved in horizontal gene transfer, thus different proteins often perform similar functions, resulting in significant redundancy [38]. This redundancy is an advantageous adaptation for *S. aureus* to sustain growth in different host environments. However, redundancy is a huge disadvantage in determining functional and physiological roles of these adhesins by mutational study designs.

In addition to adhesion, iron uptake is essential for growth and survival of *S. aureus* inside the host. Due to its limited bioavailability, *S. aureus* has evolved multiple iron acquisition systems to survive [39]. Iron in animal tissues and serum is sequestered in the form of transferrin, leaving very low amounts of free iron, approximately to the order of 10^{-24} M, which is well below the levels required by bacteria to sustain life [40]. The acquisition of iron in *S. aureus* is regulated by the Ferric Uptake Regulator (FUR). The Fur regulator can sense the bioavailability of iron, and act as a repressor to bind and inhibit the expression of target genes when iron is abundant. However, when iron is limiting, Fur repression is lifted [41]. Iron regulated surface adhesins (Isd) contain Fur box sequences upstream of their operon [42]. The Fur regulator is highly conserved in *S. aureus*, as well as other bacterial pathogens, and essential for iron sequestration [43].

The Isd proteins were first identified in 2002, by Mazmanian et al [44]. The series of Isd surface adhesins and siderophores work in a coordinated manner to transfer haem from haemoglobin across the membrane and to the cytoplasm. Isd adhesins are known to have low levels of interstrain variability, potentially due to their critical role in *S. aureus* pathogenesis [22]. Four main Isd proteins, out of a total of nine, are anchored to the cell wall by the cleavage enzymes, sortase A or sortase B. IsdA, IsdB, and IsdH contain sortase A recognition sequences, which contain a LPXTG motif, and are covalently attached to the cell wall [45]. IsdC however, has a distinct recognition sequence, NPQTN, and is anchored to the cell wall by sortase B [30]. All the other Isd proteins (DEF) are membrane localized in the ABC transporter system [46]. Multiple studies have proposed the mechanism of iron acquisition by the Isd proteins using haem receptors [33,34,44,47,48]. Conserved, 125 amino acid long, haem-binding motifs are present in all Isd proteins that are putative iron transporters [49]. IsdA, IsdB, IsdH, and IsdC contain these NEAr Iron Transporter (NEAT) motifs. IsdA and IsdC has one NEAT motif, IsdB has two, and IsdH has three. Iron acquisition through the Isd system starts with the binding of haemoglobin, myoglobin or hepta-haemoglobin by IsdB and IsdH (Figure 1-1). IsdB and IsdH can then extract haem and transfer bound haem to either IsdA or IsdE directly. The transfer of haem however from IsdA is unidirectional through the cell wall only to IsdC and then IsdE. Haem is further transferred to the bacterial cytoplasm from IsdE to IsdF, where an ABC permease transfers the molecule across the membrane using the ATP hydrolyzing ability of IsdD. Haem is degraded to iron by the Fur regulated cytoplasmic proteins; IsdG and IsdI. Studies reveal that IsdC is essential for this process and there can be no transfer of haem between IsdA to IsdE [48].

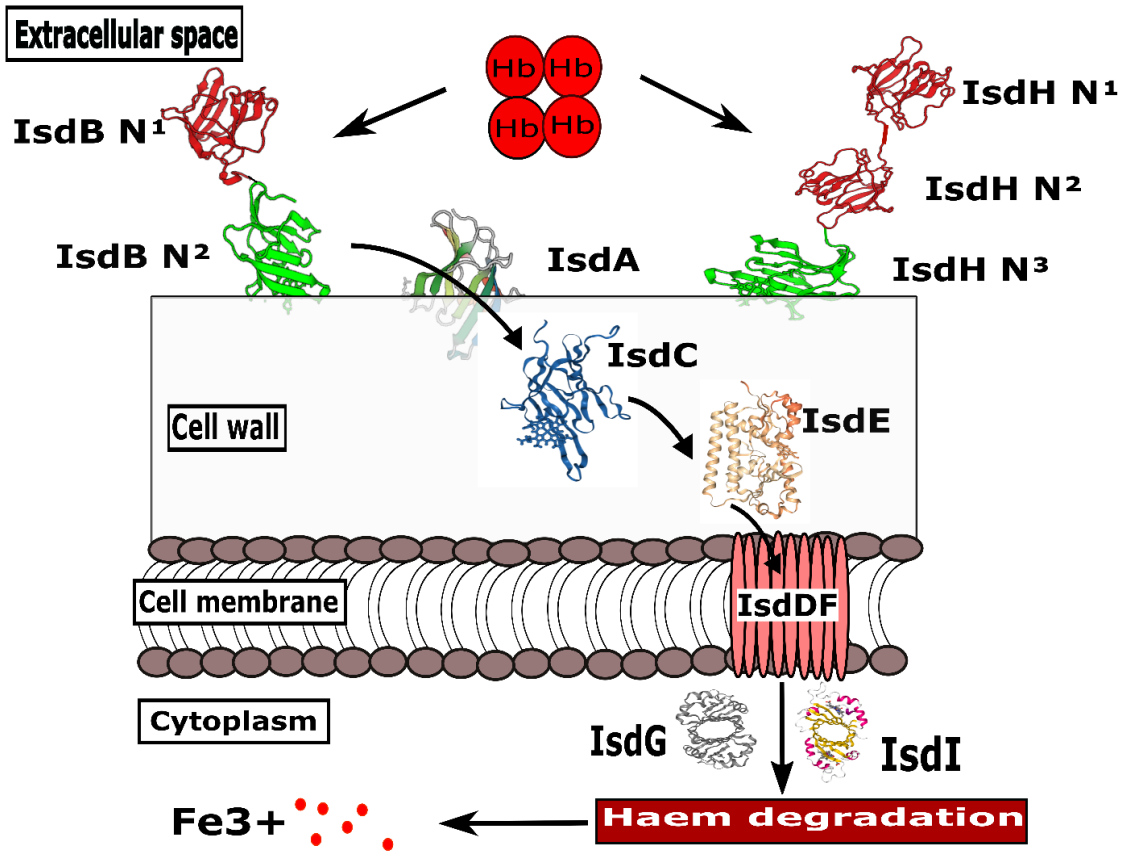

Figure 1-1. Mechanism of iron sequestration by *S. aureus*. Depiction of the mechanism of Isd-mediated iron sequestration by *S. aureus*. The first two NEAT domains of IsdH and first NEAT domain of IsdB bind to hepta-haemoglobin and meth-haemoglobin, which is then transferred to IsdA, and passed in the cell membrane via IsdC. The bound haem is then moved through the membrane by IsdDEF, which is then degraded into free iron by IsdG and IsdI. The NEAT domains of the Isd proteins shown in figure were extracted from the PDB website using the accession numbers 2H3K, 2MOQ, 2ITE, 2O6P, 2ZDO, 2ZDP [50–53].

In addition to its role in iron-acquisition, IsdA is one of the most well-characterized ECM binding adhesins [54]. IsdA has been shown to bind to FN, FG, transferrin and haem, with higher binding affinity to FN then FG [54]. The affinity of FG binding to IsdA is in micromolar dissociation constant (Kd) ranges, and 20-fold higher than that of Clumping factor A (ClfA) [34]. It is known that IsdA can bind to iron bound

ligands, however, it cannot bind to haemoglobin and hence it is not required for iron acquisition in the host [29]. IsdA is also reported to promote skin colonization by increasing the hydrophilicity or negative charge on *S. aureus* making it less susceptible to killing by the skin's natural antimicrobials [54]. Human skin is an effective barrier to infection, with epithelial cells and cross-linked keratin, and containing antimicrobial peptides and fatty acids on the surface for defense. In particular, cis-6-hexadecanoic acid in the sebum of human skin has antimicrobial activity. However, IsdA confers protection to *S. aureus* against fatty acid, and enables *S. aureus* to colonize the skin, by increasing hydrophobicity, and reducing hydrophobic interactions. Reports suggest that IsdA mutants are more susceptible to killing by human sebum, and supplementation with IsdA can fully restore the resistance phenotype [54].

Other *S. aureus* adhesins have also been well studied. The collagen binding protein Cna, the FN binding FnBP, and the FG binding ClfA, are cell wall anchored MSCRAMMs that initiate bacterial adhesion to ECM during intravascular infection. FnBP contains two very similar groups of proteins called FnbpA and FnbpB encoded by closely linked genes. *In vitro* studies suggest the importance of FnBP in adherence of *S. aureus* during infection, however, there is contrasting data for the involvement of this adhesion in endocarditis in rat models [55,56]. ClfA and ClfB are also two closely linked, but distinct adhesins. ClfA is an MSCRAMM from *S. aureus* that binds to the C-terminus of the FG γ-chain [57]. ClfA is also a well-studied virulence factor responsible for platelet aggregation and clumping of *S. aureus* in blood plasma and ClfA is a key factor in a mouse model of septic arthritis and rabbit/rat models of infective endocarditis [58]. ClfB can also bind to FG and is known to have a prominent role colonization of the

anterior nares by binding to cytokeratin [29]. The collagen binding Cna adhesin has been shown to promote adherence of *S. aureus* to cartilage [59]. The expression of Cna in an *S. aureus cna-* mutant increases its virulence [59]. Surface protein A (SpA) can bind to IgG and IgM antibodies, can inhibit opsonophagocytosis and inflammation, and is also a B cell super antigen [57]. FN-binding proteins from *S. aureus* are of particular importance, as they have the ability to bind to various types of cells in the host and can act as invasins, forming a bridge between *S. aureus* and integrin on the surface of non-professional phagocytes [60].

Other virulence factors of *S. aureus* include immune evasion molecules such as: enterotoxins, inhibitory factors and exopolysaccharides (capsule) [61]. Capsular serotypes 5 and 8 have been reported to be present in up to 70% of human and ruminant mastitis isolates, however expression is known to be variable [2]. The ability of the bacterium to colonize in tissues and disseminate throughout the host is reliant on this differential expression. Capsule production in *S. aureus*, as well as the expression of many other secreted and cell-associated virulence factors are under the control of the global regulator, accessory gene regulator (*agr*) [36] Given the importance of these virulence factors from *S. aureus* and their conserved nature, there have been many attempts to design vaccines based upon them. *S. aureus* adhesins that have been the most frequently studied as vaccine candidates for humans and animals are described below.

S. aureus Vaccine Antigens and Vaccines

IsdA

As described above, IsdA is an iron-sequestering cell wall anchored protein and an important ECM adhesin. This protein has been shown to have a critical role in nasal

colonization mediated by the NEAT domain, and the C-terminal of IsdA is responsible for cellular hydrophobicity [54,62]. IsdA is an important adhesin for both human and animal ligands [63]. An investigation of IsdA as a vaccine antigen for humans in combination with three other surface proteins IsdB, SrdD, and SrdE, was reported using the murine model. Effective protection was induced by the vaccine when mice were challenged with *S. aureus,* and modest opsonophagocytosis reported using serum antibodies [33]. In 2010, Kim et al., performed a passive immunization study using the mouse model with IsdA- and IsdB- specific antibodies. Protection against lethal intravenous *S. aureus* challenge was reported, and the group concluded that at least partial protection was achieved due to interference in pathogen's haem sequestering mechanism [64]. An immunogenicity trial in mice, conducted in the Tinker lab, was reported using cholera toxin adjuvant conjugated IsdA (IsdA-CTA_2/B). Mice were mucosally immunized with IsdA-CTA_2/B chimera and humoral and cellular immune responses detected. Results from this study revealed the induction of Th2-type immune responses that were IsdA specific, showing significant immunogenicity and supporting the potential of IsdA as a vaccine antigen [65].

ClfA

ClfA is one of the first identified conserved *S. aureus* adhesins to be considered as a vaccine candidate alone or in combination with other proteins. This surface protein has since been extensively studied and validated as a promising vaccine target and has been licensed by several companies for inclusion into vaccines [66]. In 2001, Josefsson et al. reported that mice immunized with recombinantly produced ClfA developed less severe *S. aureus* arthritis than controls. In addition, passively immunized mice with ClfA

antibodies showed protection, indicating that protection in active immunization is antibody (Ig) mediated [56]. Passive immunization with SA-IGIV (Inhibitex, Inc) polyclonal anti-ClfA antibodies were able to recognize ClfA from *S. aureus* and inhibit adherence to FG up to 95% *in vitro*. SA-IGIV also promoted efficient opsonophagocytosis *in vitro,* by opsonizing ClfA coated fluorescent beads, which further facilitated their phagocytosis by human polymorphonuclear leukocytes. Furthermore, a combination therapy, using SA-IGIV and vancomycin revealed that the clearance of bacteremia was significantly higher in combination with SA-IVIG than vancomycin alone [67,68]. Studies have revealed that there is a requirement of host FG and *S. aureus* interaction for establishing septicemia, making ClfA an extremely important vaccine target [66,69–71]. Despite these many established reports supporting the antigenicity of ClfA, a contradictory study was recently reported by Li et al.. In this study, the efficacy of anti-ClfA responses was tested preclinically in rodent and rabbit models. Immunizations with ClfA induced functional and opsonic antibodies, which were able to inhibit binding of *S. aureus* to FG, however only modest reductions in abscess formation were seen after infection with *S. aureus* USA 300 [58]. It was also reported that neither ClfA, nor passively administered ClfA antibodies, could protect against *S. aureus* bacteremia or endocarditis in rat models. Furthermore, when mice were challenged with *S. aureus* intraperitoneally after immunization with ClfA or ClfA antibodies, slight increases in bacteremia were observed in experimental murine models as compared to controls [58]. These contrasting results have been described by others as overly simplistic, with an improper focus on one antigen [72]. Thus, despite that potential challenges need to be addressed, ClfA remains a top vaccine candidate for a human

vaccine, and also for the prevention of veterinary diseases like bovine mastitis. ClfA in combination with FnbpA was recently incorporated in a DNA/protein based vaccine, that is reported to induce partial protection of mammary glands in *S. aureus* mastitis [73,74]

IsdB

IsdB is also a highly conserved cell wall anchored protein from *S. aureus* that binds to haemoglobin and aids in iron sequestration. This protein has also been studied extensively as a vaccine target and is under much scrutiny due to a recent failed human vaccine trial. In 2006, Kuklin et al., from Merck Research Laboratories, reported a clinical trial on murine and rhesus macaque models with a vaccine consisting of IsdB + amorphous aluminum hydroxy phosphate sulfate adjuvant. Results showed up to a five-fold increase in Ig titers after a single dose, and investigators described IsdB as an excellent *S. aureus* antigen, with reproducible and significant protection in animal models of infection [75]. Another report, investigating the role of cellular immunity in IsdB-mediated protection in severe combined immunodeficient (SCID) mice, determined the importance of TH17/IL-17 in IsdB mediated defense [76]. In 2014, A Phase IIB/III randomized placebo-controlled trial of the Merck V710 (Merck Sharp&Dohme Corp) *S. aureus* vaccine comprised of nonadjuvanted IsdB was conducted on 52 patients divided into 4 groups (2 placebo and 2 vaccinated) prior to elective cardiothoracic surgery. Reportedly, the vaccine failed to reduce the rate of post-operative *S. aureus* infections, and increased the mortality among patients who developed systemic *S. aureus* disease [77]. Later the pathophysiology around the results of the failed vaccine trial was explained as associated with extremely low IL2 levels in deceased patients prior to vaccination. In addition, the use of a single antigen and a focus on post-operative *S.*

aureus infection complicated study results [78]. The explanations regarding this failed vaccine trial can be due multiple other associated factors, one of them being the importance of the pre-clinical models used. Mouse immune and cardiovascular systems respond very differently to vaccines, and to *S. aureus* infection, than human systems. Current preclinical research on *S. aureus* vaccines is thus moving away from the use of rodents [79].

EsxA

EsxA is a secreted immune evasion protein and virulence factor responsible for *S. aureus* abscess formation [22]. EsxA is a part of the *S. aureus* Ess (ESX secretion) secretory pathway that contains a cluster of eight genes: *esx*A, *esa*A, *ess*A, *ess*B, *esa*B, *ess*C, *esa*C, and *esx*B [80]. EsxA has been characterized as a good vaccine antigen, and is included in vaccines alone or in combination with other antigens. EsxA is known to induce specific antibodies in sera of multi-drug resistant *S. aureus* infected patients [81]. In 2015, Bagnoli et al., from Novartis Research Laboratories reported a clinical trial using a vaccine containing five antigens (EsxA, EsxB, HlA, FhuD2 and conserved staphylococcal antigen 1A) with TH1/TH17 adjuvants [82]. The vaccine stimulated functional and opsonophagocytic antibodies and provided consistent protection in mice challenged with various strains of *S. aureus*. EsxA protein was also included in a one dose four component (4C)-*Staphylococcus* vaccine that is reported to induce protection in *S. aureus* mouse models through the combined actions of antibodies, CD4+ effector T cells, and IL-17A [83]. Overall the trials and studies on EsxA have shown promising results however, more exploration is required for its application in human and veterinary vaccines.

IsdC

IsdC is a member of the Isd family of proteins that aid in transfer of haem across the cell membrane [30]. It is also a strongly conserved protein that has potential to be used as a vaccine antigen. IsdC contains one near-iron-transporter (NEAT), domain similar to IsdA. In *S. lugdunensis*, it is shown that IsdC is required for biofilm formation in low iron conditions, for primary cell-to cell attachment and accumulation phase of biofilm formation [84]. Hence IsdC is an important virulence factor for *S. aureus* and has good potential to be used as a vaccine antigen.

Current Vaccines Against *S. aureus* Human Disease

Currently there is no licensed vaccine for *S. aureus* human infection. Two vaccines: StaphVAX (Nabi Biopharmaceuticals) and V710 (Merck Sharp&Dohme Corp) and 4 passive immunization preparations have completed clinical trials toward FDA approval. StaphVAX is composed of capsular polysaccharide 5 (CP5) and CP8 conjugated with recombinant exoprotein A (rETA) from *Pseudomonas aeruginosa* [85]. StaphVAX is reported to have failed in the reduction of *S. aureus* incidence in hemodialysis patients [86,87]. V710 provided protection against nasal carriage in a cotton rat model, increased survival of mice in a sepsis model and induced fivefold antigen-specific Ig titers [88]. Although in humans, as described above, V710 failed to reduce the incidence of post-operative *S. aureus* infection, and increased mortality in the vaccinated patients who had post-operative *S. aureus* infections [77]. The development and licensure of *S. aureus* human vaccines has been hampered by these two notable vaccine trial failures. Although there are evidences of single antigen vaccines working in

humans (*Clostridium tetani*, *Corynebacterium diphtheriae;* Tdap), it is widely agreed upon that the multi-antigen, or multivalent, approach will be essential for *S. aureus*.

Bovine Mastitis

Bovine mastitis is characterized by acute inflammation of the mammary tissues as a result of intra-mammary infection. This infection in mammary tissue is most often established by entry of the pathogen through the teat canal, commonly during the milking process, and dissemination into the mammary glands [89]. Establishing infection requires evasion of host innate immune defenses, such as: phagocytosis by neutrophils, elimination by milking, and the presence of lactoferrin in milk. Inflammation is the classical clinical sign of bovine mastitis, caused by bacterial proliferation in the mammary glands and induced by pro-inflammatory cytokines (IL-1β and TNF-α) at the site of infection [90]. Pathologically, bovine mastitis is defined by elevated somatic cells counts (SCC), at or greater than $2x10^5$ cell/ml in milk, combined with the presence of the infecting pathogen [11]. The somatic cells most often seen in mastitis are neutrophils that have migrated to the mammary glands as a first line of host immune defense [10].

Geographical region contributes to strain specificity for *S. aureus* bovine disease, however, one region will have only a few strains causing mastitis [91]. It has been reported that different quarters of the same udder can be infected with different strains of *S. aureus*, mount different levels of immune responses and have different severity of disease [92]. In addition, there are known risk factors defining the susceptibility of a cow to mastitis, such as: the stage of lactation, presence of lesions on teat skin, and the breed of cow [93–96]. The route of transmission is also an important consideration that can contribute to progressive colonization and severity of infection [97]. It is known that if *S.*

aureus contaminates the teat orifice, it multiplies there and enters the teat canal for progressive colonization and dissemination. This infection occurs mainly by milking machine, and due to intra-mammary changes in pressure causing the bacterium to be transferred to the teat sinus [98].

Infection of bovine mastitis can remain in the subclinical, or chronic, phase indefinitely, or it can alternate between clinical and subclinical disease. The progression of bovine mastitis is defined by three stages: *1)* pathogen invasion, *2)* infection and *3)* inflammation [99]. In the invasion stage, the pathogen moves up the teat canal. In the infective stage, bacteria bind and adhere to mammary cells to establish infection in the gland cistern, divide and proliferate. In the final inflammatory stage, there is an increase in SCC, more cytokines are released and clinical signs of disease appear. As shown in Figure 1-2, the mammary glands of cows are anatomically designed to function as barriers of inoculation and progression of pathogens. The teat canal of the mammary gland is an important barrier and first line of defense against intra-mammary infection. Teat sphincter muscles in the canal keep the canal closed, so that the mammary gland is isolated from any potential infection of bacteria [100]. However, damage to the teat canal and teat skin can increase the risk of mammary gland infection. The surface of the teat canal contains fatty acids and keratin proteins that acts as a bacteriostatic agents [101]. Additionally, cationic proteins present in the teat canal can bind to pathogenic bacteria, increasing their susceptibility to the osmolarity of the canal [102]. If bacteria penetrate the teat canal, they encounter the innate and adaptive immune responses, including: neutrophils, macrophages and lymphocytes [40]. The proinflammatory cytokines TNF-α and IL-1β are also released at the site of infection, stimulating the bactericidal activity of

neutrophils and macrophages, which in turn produce leukotrienes and prostaglandins to increase the local inflammatory reaction [103]. Epithelial cells of the mammary gland also play an important role in neutrophil recruitment to the infection site, adherence of bacteria and interaction with bacterial toxins [104]. Thus the innate and adaptive immune responses perform crucial roles in the pathophysiology of bovine mastitis, and these responses are regulated mostly by pro-inflammatory cytokines, increases in macrophage and neutrophil bactericidal activity, recruitment of neutrophils to the site of infection, and maturation DCs [105].

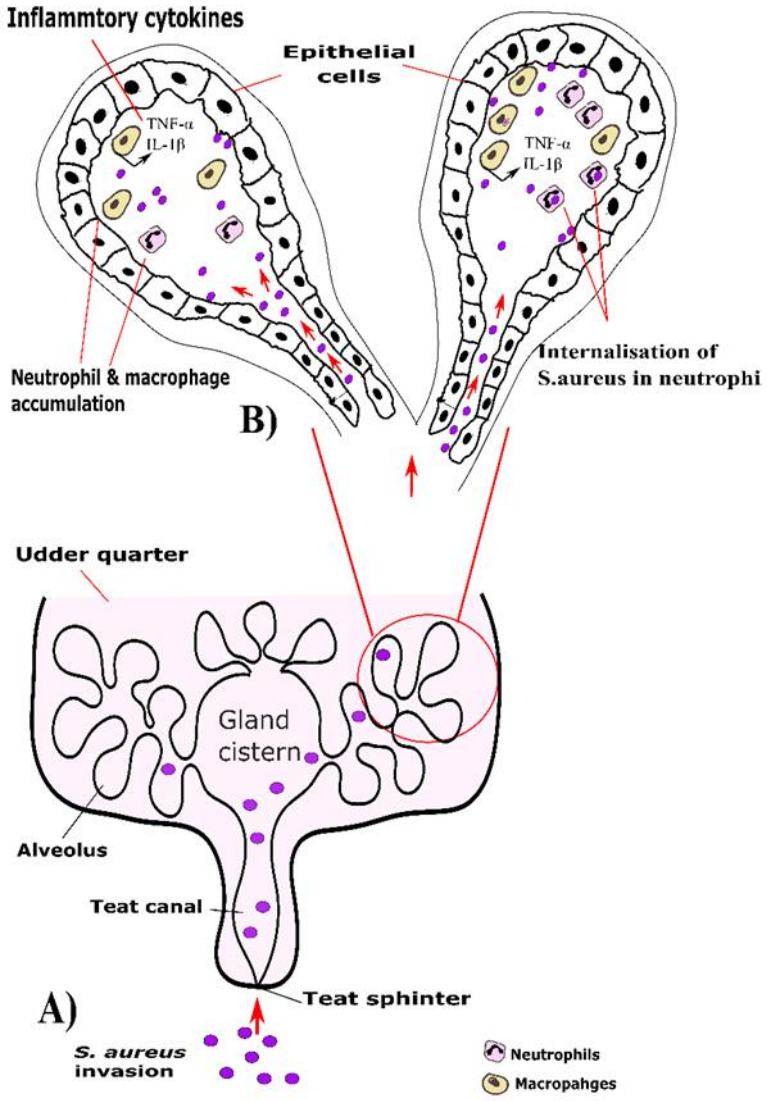

Figure 1-2. Inflammatory model of *S. aureus* caused bovine mastitis. A) The infective stage of *S. aureus* transfer through the teat canal and its colonization and movement through gland cistern and alveolus. B) The inflammatory phase, highlighting the magnified section of alveolus. The binding to *S. aureus* to mammary epithelial cells is shown, in addition to the internalization of *S. aureus* in macrophages and neutrophils plus the induction of inflammatory cytokines IL-1β and TNF-α.

Physiological attributes associated with the mammary glands also make them more susceptible to *S. aureus* colonization and infection. Despite the high concentration of iron-binding proteins like lactoferrin, milk is an excellent, nutrient-rich, medium for

bacterial growth. In addition, the mammary glands of cows have reduced hydrolytic enzymes and phagocytic activity, to prevent the engulfment of milk components like proteins and fats [106]. This lack of phagocytosis prevents the engulfment of pathogenic bacteria as effectively as seen in other tissues of the body. In the absence of a strong phagocytic response, *S. aureus* adhesins can bind to the mammary gland epithelium and macrophages, to induce bacterial uptake and intracellular survival inside these cells [107]. Casein protein is in abundance in mammary glands due to the prevalence of milk; this protein can coat the surface of neutrophils disabling their pseudopodia and hence their ability to translocate [108]. Complement proteins and opsonizing antibodies are low in abundance in milk and mammary tissues, which also helps bacteria to colonize and cause infection without stimulation of the complement system or opsonization. Lastly, it is reported that during the bovine pre-and post-parturition period there is a functional impairment of lymphocytic and neutrophilic activity due to stress, hormonal changes and lactation [109].

The pathogenicity of *S. aureus* in the mammary gland is a combined effect of virulence factors, specifically the ones that help in adhesion to host tissue, those that promote tissue damage and those that promote intracellular survival and evasion of host immune system. The virulence of some strains of *S. aureus* is dependent on growth phase and nutrient availability [110]. As described above, *S. aureus* produces a number of important proteins that bind to FN, FG and collagen, and these are involved in colonization of the udder. Lack of the adhesin, FnBP, causes a delay in internalization of *S. aureus* in mouse mastitic epithelial cells compared to the wild type [55]. Extracellular fibronectin binding protein (Efb) is studied for its immunogenicity in subclinical bovine

mastitis, in combination with FnbpA and ClfA, and shown to stimulate a strong humoral and mucosal immune response in vaccinated animals [111,112]. Eap/map virulence factor binds to ICAM-1 located at the surface of endothelial cells and blocks neutrophil binding to these cells [113]. Other virulence factors that contribute to disease progression and inflammation during mastitis include: exotoxins, bacterial super antigens, proteases and anti-phagocytic factors.

The adhesive ECM molecules like FN, collagen, FG, laminin, vitronectin and elastin are present in the mammary gland and interact with bacterial adhesins, aiding in adherence and colonization [100]. FN exists in soluble and insoluble forms in the matrix and serves as a bridge between *S. aureus* and integrin-β1. This bridge, or contact, of *S. aureus*-to cell-to ECM is important to prevent apoptosis. After adhesion is established by *S. aureus* to mammary epithelial cells or MacTs, the integrin receptor starts the cascade of transduction events leading to uptake of bacteria [114,115]. Adherence to FN is essential *S. aureus* infection, and it has been demonstrated that using anti-FN antibodies, can significantly reduce adherence of *S. aureus* to epithelial cells [116,117]. *S. aureus* FN-binding adhesins include FnbpA and IsdA, and these represent good bovine mastitis vaccine targets.

Treatments and Prevention of Bovine Mastitis

Bovine mastitis results in economic losses to dairy farmers due to poor milk yield, veterinary expenses, culling and discarded milk [118]. If bulk milk tank SCC is 400,000 cells/ml, or higher, it is considered unfit for human consumption [119]. *S. aureus*, clinical or subclinical infection, is a major contributor to high SCC in bovines. Currently bovine *S. aureus* is treated with antibiotics, however, the cure rates are variable and often low

[120]. There are several parameters which determine the success of antibiotic treatment and cure. These include: higher than average increase in SCC, age of the cow, duration of infection, quarters of udder infected, resistance of strain, and bacterial load [121]. It is known that any quarter of the cow udder can be infected with *S. aureus*, and reports suggest that hind quarters have higher cure rates than front quarters [122]. Antibiotics found to deliver higher cure rates include: amoxicillin, erythromycin, cloxacillin, and pirlimycin [123]. The efficacy of antibiotic therapy for bovine mastitis varies between 4% to 90%, depending upon the causal agent. *S. aureus* caused mastitis is among the most difficult to treat, with cure rates lower than 25% [124]. The unique ability of *S. aureus* to internalize in mammary gland epithelial cells or neutrophils/ macrophages affects the efficacy of antibiotics due to restricted access of innate and adaptive immune cells [125]. Furthermore, *S. aureus* is known to form biofilms in the udder, which also contributes to antibiotic resistance [126]. In addition to lower cure rates, there are other disadvantages of antibiotic use. Overuse or misuse of antibiotics can lead to the emergence of new antibiotic resistant strains of bacteria, which risks non-treatable infection, resistant strains in the food chain, and possibly the presence of antibiotic residues in cow milk. The cost of antibiotics and its long-term use impact animal health, agricultural economics and public health.

Prevention of *S. aureus* mastitis with an effective vaccine would be a much preferable option over treatment. Developing a vaccine has proven to be difficult however, and those currently available provide limited efficacy and protection. Currently, there are two bovine *S. aureus* vaccines: Mastivac I (Ovejero Laboratories) and Lysigin/Somato-staph (Boehringer Ingelheim Vetmedica, Inc.). Mastivac, is a

commercially available vaccine, composed of fragments from three bovine clinical staphylococcal strains (VLVL8407; ZO3984 and BS449). This vaccine's compositions are exosecretions of strains VL8407 and bacterial fragments of strains of ZO3984 and BS449 mixed with either incomplete Freund's adjuvant (IFA) or ISA (SEPPIC, d'Orsay, Paris, France) [106,127,128]. Mouse trials using this vaccine did show immunogenic properties in addition to protection against homologous and heterologous *S. aureus* challenge, however, in bovine trials, only a slight reduction in new *S. aureus* udder infection was observed [106]. Lysigin (Boehringer Ingelheim Vetmedica, Inc.) is another commercially available vaccine for bovine mastitis, it is composed of a lysed culture of antigenic polyvalent somatic antigen containing *S. aureus* phage types I, II, III, IV and capsular polysaccharides [129]. This vaccine is capable inducing anti-staphylococcal antibodies in bovines, however it stimulated insufficient opsonizing antibodies in milk to facilitate phagocytosis and clearance of *S. aureus* from the mammary gland [130]. Induction of opsonizing antibodies is an important attribute of an effective *S. aureus* vaccine [131].

While current vaccines are reported to reduce the incidence of *S. aureus* caused bovine mastitis, they were not capable of complete prevention or elimination of transmission, and are not commonly used by dairy farmers in the U.S. An effective vaccine for *S. aureus* caused bovine mastitis thus still remains a top priority for the dairy industry. As stated above, the success of an *S. aureus* vaccine will depend upon the inclusion of multiple relevant antigens, and importantly, the success of such a vaccine will also depend upon induction of an effective immune response. For subunit based vaccines, the use of the correct adjuvant, to aid in proper antigen presentation and

enhancement of the immune response to make memory responses and induce long term efficacy, is essential. As described below, cholera toxin (CT) is a unique and promising vaccine adjuvant that may be effective to prevent bovine mastitis.

Cholera Toxin as an Adjuvant for Subunit Vaccines

Bacterial enterotoxins like: CT, heat labile toxin (LTI, LTIIa, LTIIb) and shiga toxin (Stx1 and Stx2), have long been studied as vaccine adjuvants. CT is a secreted AB type enterotoxin from *Vibrio cholerae* that is responsible for the watery diarrheal clinical outcome of disease. CT is composed of a larger heteromeric A subunit (240 amino acids; MW 28 kD) located centrally, and five homomeric B subunits (103 amino acids; MW 11 kD) located peripherally [132]. The A subunit consists of two domains (Al and A2) and the upper A1 domain is held above the plane of the doughnut-shaped pentameric B subunits by the tethering A2 domain. The A1 domain is essential for enzymatic activity and conducts ADP ribosylation within the host cell. The five B subunits act as a receptor for cell entry by high-affinity binding to GM1 ganglioside present at the cell surface [133]. GM1 gangliosides are expressed ubiquitously on the surface of antigen presenting cells (APCs), macrophages, dendritic cells (DCs), natural killer cells (NK cells), epithelial cells and B cells [134].

V. cholerae colonizes the intestinal epithelium, and once attached, the bacterium secretes toxin. CT is activated by host proteases (“nicking”) prior to entry into the cell [135]. As shown in Figure 1-3, once bound to host GM1, CT is transported into the endosomes, and moved in a retrograde path through the Golgi apparatus to the Endoplasmic Reticulum. The ER considers CT an incorrectly folded protein and degrades it using ER associated degradation (ERAD) [136]. Once degradation occurs the A subunit

crosses the ER membrane into the cytosol. The A1 subunit retains its native state in the cytosol and enzymatically triggers ADP ribosylation of Gαs. This results in an irreversible increase in the intracellular concentration of cAMP, activation of PKA (protein kinase A), and phosphorylation of the cystic fibrosis transmembrane receptor (CFTR) Cl^- channel. The activation of CFTR leads to efflux of chloride ions out of the cell, causing massive water efflux, and the severe watery diarrhea that is characteristic of cholera disease [137].

The translocation and stimulatory activities of CT toxin also make it an optimal vaccine adjuvant, especially for an intracellular and mucosal pathogen like *S. aureus* [138]. CT is immunogenic and is known to stimulate systemic and mucosal humoral and cellular responses [139]. CT can also potentiate the immunogenicity of most antigens, whether these are linked to or simply admixed with the toxin, provided that the antigen is given at the same time and at the same mucosal surface as the toxin [140]. Specifically, CT has been reported to induce: (i) the activation and permeability of the intestinal epithelium, leading to higher antigen uptake, (ii) increased antigen presentation by antigen presenting cells (APCs), (iii) the promotion of isotype differentiation of B cells to induce secretory IgA production, (iv) the promotion of stimulatory and inhibitory effects on cytokine production and (v) the upregulation of co-stimulatory molecules, such as MHC class II, to promote T cell differentiation.

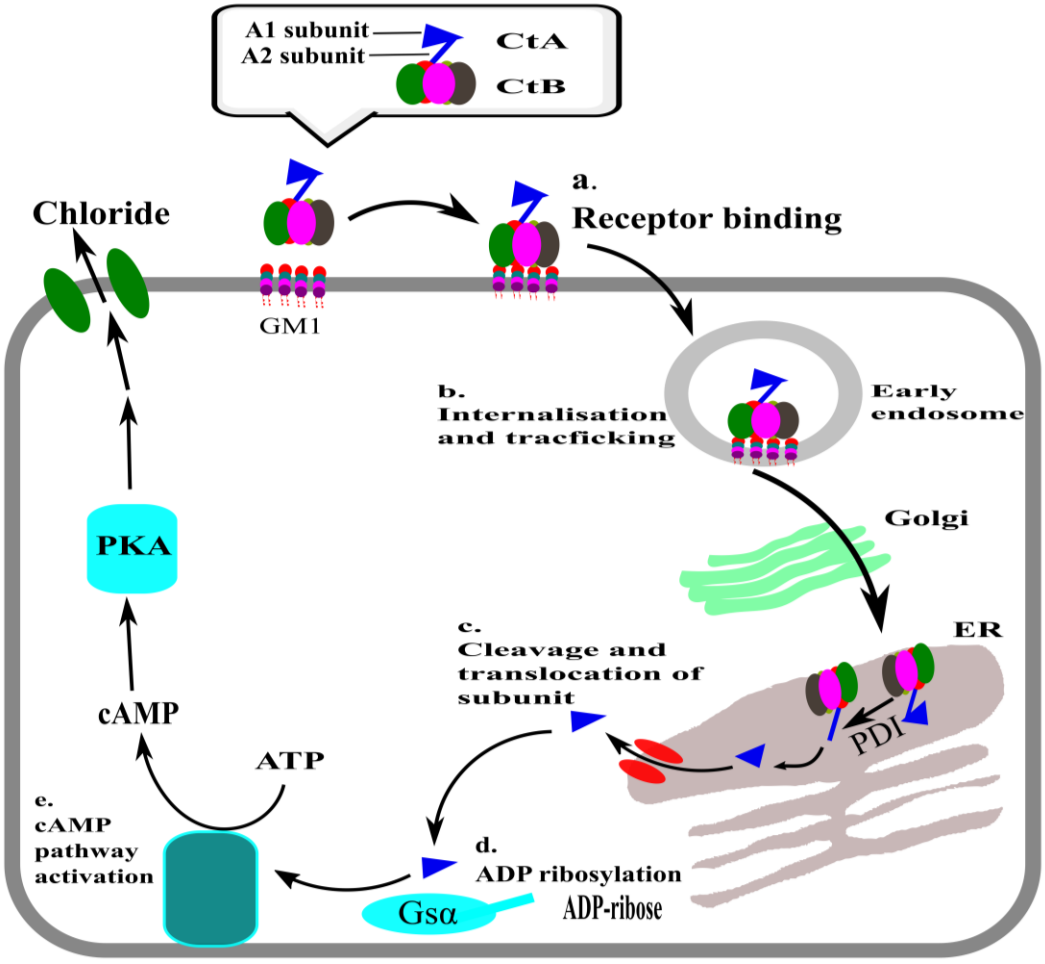

Figure 1-3. Model of CT entry and trafficking. CT binds to the B subunit by GM1 gangliosides. It then traffics through the early endosome to the Golgi apparatus and is eventually delivered to endoplasmic reticulum (ER). Once in ER the A1 subunit is unfolded and retro translocated by the ERAD pathway to the cytosol. Once in cytoplasm A1 subunit refolds into native form and activates adenyl cyclase and the increase in cAMP causes chloride secretion.

Native Ct: Immunostimulatory Effects

Induction and potentiation of APCs is one of the most important characteristics of an adjuvant [141]. Native, toxigenic CT has been shown to play a role in the maturation and induction of DCs in humans and mice [142]. B cells also play an important role establishing immunity by the production of antibodies and antigen presentation, it has been shown that when B cells are treated with CT they increase the induction of co-

stimulatory signals required for activation of T cells by APCs [143–145]. Antigens conjugated to CT can be mucosally delivered faster and more efficiently to APCs, and this includes DCs, macrophages and B cells [140]. The stimulatory effects CT has on antigen presentation as well as the increase in the activation and migration of DCs suggests that CT is able to elicit both innate and adaptive immune responses through its actions on APCs. CT is also known to enhance T cell proliferation by increasing IL-12 and IFN-γ cytokines when coadministered with an antigen [142]. *In vitro* studies indicate that a very small amount of antigen is required to stimulate a proliferative T-cell response when conjugated with CT [146]. Although the polarity of immune responses generated by CT is a matter of debate, some studies indicate that CT-treated DCs prime the naïve T cells *in vitro* and drive them towards a Th2 phenotype. However, a few studies report that the toxin induces a mixed Th1/Th2 type of immune responses [147].

The success of any vaccine relies on the stimulation of unique antibodies against the antigen of interest and prevalence of those antibodies as an immunological memory [148]. B-cell antigen presentation is an important criterion in establishing an effective immune response with vaccines [149]. Current adjuvants have been chosen and designed based on their ability to elicit a cellular responses, specifically their abilities to induce a Th1, Th2, or Th17 cell response, as well as the amount of Ig produced [150]. CT has long been reported to induce antigen-specific IgG and IgA to co-administered antigens, however, little information exists regarding the ability of CT to influence immunologic memory [148]. More recently, Bemark et al., reported the memory-inducing capabilities of CT on B cells in mice [151]. Another report has indicated that CT supports the

development of immunologic memory by proliferation and activation of lymphocytes within the (GCs) germinal centers [152].

Non-Toxic CT Derivatives

While native CT is the gold-standard, non-toxic CT derivatives have also been found to retain significant adjuvant activity. CT as compared to CTB showed enhanced antigen-presenting capabilities, suggesting a supportive role of toxicity on antigen delivery [140]. However, it has been reported that B cells treated with CTB alone experienced an increase in the co-stimulatory signal required for T-cells activation. A recent study by Lundgren et al., showed that specific memory IgA antibodies against CTB were induced in the intestine by the oral CTB-based cholera vaccine, Dukoral [148]. A non-toxic purified monomeric CTA1-DD (adjuvant) fusion protein, that contains the ADP-ribosylating enzyme from CT combined with the immunoglobulin binding domain of *Staphylococcus aureus* protein A, was shown to directly stimulate mast cells and to release TNF-α [153]. Mast cells are a promising target for adjuvants as they are located on the skin and throughout mucosal surfaces and act as a first line defense for many pathogens. Their location within the mucosal tissue combined with their newly found role for altering adaptive immune responses make them an interesting target for adjuvants [154]. This team also found that immunized mice had Ig half-lives that were two to three times longer than Ig from mice immunized with other adjuvants. Interestingly, the increase in Ab half-lives correlated with an increase in germinal centers (GC) activity [151].

While CTB clearly retains significant adjuvanticity, the mechanism of antigen conjugation or interaction, route of administration, and concentration of antigen/adjuvant

notably affect the immunstimulatory outcome. It has been reported that CTB directly conjugated to an antigen and administered orally, nasally or sublingually at high concentrations, can induce tolerance more than the administration of antigen alone [155]. CTB has also been reported to induce autoimmunity to self-antigens and tolerance to autoimmune antigens, however, this seeming paradox is not currently well understood [156,157]. The most recent studies using CTB adjuvant have focused on the ability of CTB conjugates to act as proinsulin vaccines to suppress diabetes [158,159]. It remains to be determined if this versatility will be a benefit for the use of CTB-based adjuvants or a potential disadvantage.

CTA_2/B chimeras are toxin-based vaccines plus adjuvant that possess the GM1 binding ability of the B subunit and the ER targeting (KDEL) motifs on the A_2 subunit. These molecular possess the unique adjuvant qualities of CTB and are non-toxic [65]. The CTA_2/B holotoxin-like molecule was first constructed in 1992 to develop an adjuvant that could be linked to the antigen of interest [160]. These molecules have since been reported as potential vaccines by a number of groups [161–170]. The Tinker laboratory has reported the construction a and purification of CTA_2/B with the LcrV protective antigen from *Yersinia enterocolitica* and *Y. pestis*, making LcrV–CTA_2/B chimeras [168]. This chimera showed the uptake and trafficking of the LcrV antigen like CTA_2/B *in vitro*, reflecting the antigen-delivery ability of the CTA_2/B adjuvant [171]. CTA_2/B chimeras have also been used as mucosal adjuvants in a subunit vaccine against *S. aureus*. In this report, the Tinker Laboratory developed an IsdA-CTA_2/B chimera that stimulated significant humoral immune responses in mice after intranasal delivery [161] . Furthermore, a DIII-CTA_2/B chimera, targeted against the West Nile Virus, was

constructed and found to induce antigen-specific IgM and IgA responses in mice [170]. In summary, the current data suggests that CTB and CTA_2/B are highly immunogenic and can enhance specific immune responses when conjugated or co-expressed with antigens. However, further investigations to explore the mechanisms of action of these adjuvants including the potential to induce tolerance/autoimmunity, will allow more effective use of these molecules in future vaccines.

Summary and Objectives

Many studies and a few failures in human and veterinary vaccines have led to a growing belief that an efficacious vaccine for *S. aureus* infection will be difficult or impossible to achieve. Unraveling the pathogenic and evasion mechanisms of *S. aureus* will be important for the use of critical factors involved in these processes in vaccines. Although there is evidence with other pathogens that a single antigen vaccine can be effective, a multi-antigen vaccine approach will be essential for *S. aureus*. This is due to the complex pathogenicity and expression of many different virulence factors. An efficacious vaccine for *S. aureus* mastitis should include multiple highly conserved antigens to protect against various strains, and a safe and immunogenic adjuvant. Importantly, the vaccine must induce opsonizing antibodies, as well as cellular responses and be able to target mucosal surfaces.

The overarching hypothesis of this research is that a *mucosal, enterotoxin-based vaccine containing multiple relevant antigens will protect cows against S. aureus mastitis.* To begin to address this hypothesis, the major aim of our research was to design a mucosal cholera toxin (CTA_2/B) adjuvant based mucosal vaccine for *S. aureus* caused

mastitis. To achieve this aim, my dissertation work was focused on three distinct objectives:

Objective 1: Expression, immunogenicity and variation of iron -regulated surface protein A from bovine isolates of *Staphylococcus aureus*

IsdA is an important adhesin, virulence factor and invasin for *S. aureus.* It is reported to be conserved in human isolates and a good vaccine candidate. The determination of *isd*A genetic conservation and expression in bovine *S. aureus* is critical for its use in a bovine vaccine. The outcomes of this objective were threefold. These studies: *1)* determined *isd*A conservation and IsdA expression from bovine *S. aureus*, *2)* identified and characterized variants of IsdA and the binding capacity of these variants to FN and FG, and *3)* identified the opsonizing ability of IsdA specific antibodies. Opsonophagocytosis is a critical property of antibodies essential for *S. aureus* vaccine efficacy.

Objective 2: Immunogenicity of mucosal *Staphylococcus aureus* vaccine for bovine mastitis

There is currently no effective vaccine available to prevent bovine mastitis. To develop a vaccine, the two conserved adhesins, IsdA and ClfA from *S. aureus* were used in combination with CTA_2/B adjuvant. The immunogenicity of this chimeric vaccine was determined by two bovine trials completed during the dry off period. Milk and serum from cows were used to detect humoral and cellular immune responses in addition to detecting the opsonizing ability of vaccine-induced antigen specific antibody. The outcomes of this objective included: *1)* the mucosal vaccine (IsdA-CTA_2/B and ClfA-CTA_2/B) was able to stimulate humoral and cellular immune responses with largely a

Th2 bias, and 2) the antibodies stimulated against IsdA antigen were specific and capable of opsonophagocytosis.

Objective 3: Immunoproteomics to identify *Staphylococcus aureus* antigens expressed in bovine milk during mastitis.

S. aureus has many virulence factors required to adhere, invade, cause infection and aid in survival and proliferation. A multivalent vaccine that can be target the diseases associated with multiple strains and serotypes of the *S. aureus* will be more effective in preventing *S. aureus* caused mastitis. In this objective, two-dimensional electrophoresis (2DE) combined with mass-spectrometry was used for the detection of immunogenic virulence factors and surface proteins from *S. aureus.* The outcomes of this objective included: *1)* the identification of 8 *S. aureus* surface proteins that are virulence factors and were immunogenic in bovine milk *in vitro,* and 2) the evaluation of two proteins, IsdC and EsxA as strong *S. aureus* vaccine candidates for incorporation into a bovine mastitis vaccine.

CHAPTER TWO: EXPRESSION, IMMUNOGENICITY AND VARIATION OF IRON-REGULATED SURFACE PROTEIN A FROM BOVINE ISOLATES OF *STAPHYLOCOCCUS AUREUS*

Abstract

Staphylococcus aureus iron-regulated surface protein A (IsdA) is a FG and FN adhesin that also contributes to iron sequestration and resistance to innate immunity. IsdA is conserved in human isolates and has been investigated as a human vaccine candidate. The studies presented here describe the expression of *isd*A, the efficacy of anti-IsdA responses and the existence of IsdA sequence variants from bovine *Staphylococcus*. Clinical staphylococci were obtained from U.S. dairy farms and assayed by PCR for the presence and expression of *isd*A. *isd*A-positive species from bovines included *S. aureus, S. haemolyticus* and *S. chromogenes*. Immunoassays on bovine milk and serum confirmed the induction and opsonophagocytic activity of anti-IsdA humoral responses. The variable region of *isd*A was sequenced and protein alignments predicted the presence of two main variants consistent with those from human *S. aureus*. Mouse antibodies against one IsdA variant reduced staphylococcal binding to FN *in vitro* in an isotype-dependent manner. Purified IsdA variants bound distinctly to FN and FG. These findings demonstrate that variability within the C-terminus of this adhesin affects immune reactivity and binding specificity, but are consistent with the significance of IsdA in bovine disease and relevant for vaccine development.

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most prevalent pathogens causing contagious subclinical and clinical bovine mastitis. Bovine mastitis is an inflammatory disease of mammary tissues in cows, often represented by marked increase in somatic cell count (SCC) [11,172–174]. Several pathogenic bacteria are known to cause bovine mastitis; however, *S. aureus* causes persistent, difficult or impossible to treat infections greatly impacting animal health [172–175]. This is the most economically important disease for the dairy industry, largely due to loss of milk and the cost of treatment [176]. In the U.S. alone, the economic loss by bovine mastitis is estimated to be approximately 100-200$/cow [14,15,177,178]. The main treatment strategy adopted currently to treat bovine mastitis is the use of antibiotics and management practices at dairy farms. Excessive use of antibiotics is both a costly and risky option as it may lead to the emergence of new antibiotic resistant strains. Efforts have been made for treating and managing this disease, however, prevention by vaccination is the best approach. Effective protection with a vaccine for a variable pathogen like *S. aureus* will require a vaccine design which includes conserved virulence factors and determination of its antigenic variation.

S. aureus possess a repertoire of virulence factors and surface adhesins, that enables its survival in the varied physiological conditions of the host [63,179]. Iron surface determinant (Isd) proteins are a class surface adhesins, that are involved in iron acquisition and ECM binding [27,180]. Iron is a critical nutrient for survival of *S. aureus* and its uptake is regulated by the FUR (ferric uptake regulator) regulator, in low iron conditions [41]. The NEAT (near iron transporter) domain of Isd proteins is involved in

haem binding from haemoglobin, for uptake into the bacterial cytosol [47,62]. As shown in Figure 1-1 Chapter 1, the transfer of haem from membrane to cytoplasm is performed by four or more Isd proteins (IsdA, IsdB, IsdC, IsdH) [44,181]. IsdA is one of the most conserved of these proteins [45]. It is known to be critical for nasal colonization in humans and provides hydrophobicity to the cells through a cell wall binding (LPTGX) motif, aiding in colonization of *S. aureus* on the skin [29,182]. The NEAT domain in IsdA is known to play a role in binding of IsdA to FN, however, the significance of the C-terminal of IsdA in ECM binding is still not well described [25,77]. The C-terminus of IsdA is involved in surface hydrophobicity, that confers resistance to the antimicrobial peptides and fatty acid of the human skin [54]. These reports indicate that IsdA is an important virulence factor for *S. aureus* that may contribute to infection in the bovine udder.

The goals of this study were three-fold: *1)* to determine the presence of *isd*A in bovine isolates of *Staphylococcus*, *2)* to determine the expression of IsdA *in vivo* as determined by qPCR and anti-IsdA immune responses, and *3)* to identify IsdA sequence variation. Two main sequence variants were distinguished and the difference in binding specificities and immune activity of the two distinct isdA allelic variants was defined. The results of this study support the incorporation of the IsdA antigen in a multivalent bovine vaccine, however, it is acknowledged that variability of this antigen will be an important consideration for vaccine design.

Materials and Methods

Bovine Milk, Serum and Staphylococcal Collection

Blood and quarter milk were taken from observationally healthy lactating Holstein cows from Dairy 1 (D1; *n* = 53) and Dairy 2 (D2; *n* = 50) in the Northwest USA. All bovine sample collection protocols were pre-approved by the University of Idaho Institutional Animal Care and Use Committee (IACUC). SCCs were determined using the Delaval method (Tumba, Sweden). Milk (100 μl) was plated on mannitol salt agar (MSA) and MP2 Agar (Udder Health Systems Inc., Tacoma WA, USA) and cultured at 37◦C for 24 h. Samples with yellow growth on MSA plus growth in the absence of esculin hydrolysis on MP2 were presumptive staphylococci. Cultures were incubated in lysis buffer (20 mM Tris Cl, 2 nM EDTA, 1.2% Triton X-100 + 20 mg/mL lysozyme, pH 8.0) for 30 min at 37◦C prior to DNA extraction (DNeasy, Qiagen, Valencia, CA, USA). Milk was centrifuged to remove fat layers. Blood was allowed to coagulate at room temperature. Milk and serum were diluted 1:10 in protease inhibitor (1:100 Thermo-Fisher HALT, 5% glycerol in 1× PBS) and frozen at −20◦ prior to ELISA. An additional 25 bovine *Staphylococcus aureus* isolates from clinically mastitic cows on 15 farms in six US states were obtained (Udder Health Systems, Inc.) These strains had been analyzed by mass spectrometry (MALDI). Species identification of all staphylococci was confirmed by 16S rRNA sequencing (GE Healthcare, SeqWright, Houston, TX, USA). Gene presence/absence was determined by electrophoresis of PCR performed under optimized conditions, and amplicon sizes are as indicated (Table 2-1).

Table 2-1. Bacterial strains, plasmids and primers used in this study

Bacterial strains		
	Genotype or characteristics	**Reference or source**
E. coli TE1	Δ*endA* derivative of TX1	(8) (8)
S. aureus Newbould 305	Bovine clinical isolate	(9)
S. aureus MRSA USA300	Human clinical isolate	(10)
S. aureus MRSA252 #75	Human clinical isolate	(11)
Plasmids		
	Gene	**Vector**	**Reference or source**
pBA015	*isd*A (MRSA252)	pET40(b)	(11)
pBA009A	*isd*A (MRSA252)	pTRCHisA	(11)
pCK001	*isd*A (Newbould)	pTRCHisA	This study
Primers			
	Gene	**Amplicon (bp)**	**Reference or Source**
FW CGGTTCAACCAAAACCTGCT RV GCGAAGGCAACTGTGCTAAT	*isd*A	380	This study
FW CAAATGGCGAAGCACAAGCAG RV CAAATGGCGAAGCACAAGCAG	*isd*B	138	This study
FW AGCAGCACTGCAACAAATCC RV CCATGGACCATTGGATCT	*isd*H	557	This study
FW CCTATGCCAGTAGCCAATGTC RV GCACCAAGCAGGTTATGTC	*clf*A	318	This study
FW GTGAAAAACAATCTTAGGTAC RVTATCAATAGCTGATGAATCCG	*fnbp*A	1750	(12)
FW GCGATTGATGGTGATACGGTT RV AGCCAAGCCTTGACGAACTAAAGC	*nuc*	270	(13)
FW GGGATCATAGCGTCATTATTC RV AACGATTGTGACACGATAGCC	*mec*A	527	(14)
FW GGCCGTGTTGAACGTGGTCAAATCA RV TACCATTTCAGTACCTTCTGGTAA	*tsta*G	370	(15)
FW 8F AGAGTTTGATCCTGGCTCAG RV 534R ATTACCGCGGCTGCTGGC	16s rRNA	530	(16)

RT-PCR, ELISA, Western Blot and Opsonophagocytosis

Total RNA was isolated from milk as described [183]. Briefly, milk was centrifuged at 3000 × *g* for 15 min at 4◦C and pellets washed (1× PBS + 0.5 mM EDTA). RNA was extracted (RNeasy, Qiagen), with an additional DNase (Promega, Madison, WI) treatment (1U/100 μL, 30 min). cDNA was transcribed as described by the manufacturer (High Capacity cDNA RT kit, Thermo Fisher) and included no RT controls. 16S rRNA and *isd*A PCR were conducted on 2 μL of cDNA with primers (Table

1) and GAPDH control. PCR (15 μL) was analyzed on 1% agarose with positive (Newbould 305) and negative (no template). For milk and serum ELISA, a HIS-IsdA fusion (pBA015, Table 2-1) was purified with cobalt chromatography as described [161]. Plates (Nunc, Thermo-Fisher) were coated with 50 μL of 10 μg/mL IsdA, and incubated for 12 h at 4◦C followed by washes (1× PBS + 0.05% Tween 20). Blocking buffer (1× PBS + 1% skim goat milk) was added for 2 h at 37◦C. Twofold dilutions, starting at 1:2 for milk and 1:10 for serum, were added and incubated at 4◦C for 12 h prior to washing and addition of HRP-anti-bovine IgG (1: 10,000 Bethyl Laboratories, Montgomery, TX, USA). Samples were developed with tetramethylbenzidine (TMB One, Thermo-Fisher). Endpoint titers were defined as the reciprocal of the dilution giving an OD of 0.2. *Staphylococcus* + and *isd*A ± cows used in ELISA are shown (Table S1, Supporting Information). Ten cows from each farm, with both high and low SCC, and negative for *Staphylococcus* by culture, were also assayed (*Staphylococcus–isd*A–). ELISAs are by quarter for milk and by cow for blood. For westerns, IsdA (pBA015, 62 kD) was separated on 12% SDS-PAGE, transferred to nitrocellulose strips and incubated in blocking buffer (0.05% Tween-20 + 5% skim milk + 1× PBS) before probing with milk at 1:500. HRP-antiboxing IgG (1:5000 Bethyl Labs, Montgomery, TX) was added and strips were developed with Immobilon Western HRP substrate (Millipore, Billerica, MA, USA). Opsonophagocytosis (OSP) was performed as described by [131] with the addition of purified IsdA. IsdA (pCK001) was purified using cobalt as above for pBA015. Equal volumes of 50 μg heat-inactivated serum were mixed with 50 μg purified IsdA and incubated for 1 h at 37◦C. Equal volumes of *S. aureus* Newbould 305 (2×10^6) and bovine PBMCs (2×10^6) were added to the reaction and incubated at 37◦C. At 60 min,

cells were plated on MSA in triplicate. Results are reported as bacterial survival in CFU/mL and compared to survival without PBMCs.

Table S1. Staphylococcus species from Dairies 1 and 2

CowID	*QTR*	*MP2*[a]	*CT*[b]	*isdA*[c]	*Sequence ID*[d]
D1-02	FL	γ	-	-	*S.agnetis*
D1-07	BR	γ	-	-	*S.xylosus*
D1-07	FL	γ	-	+	*S.chromogenes*
D1-07	FR	γ	-	-	*S.haemolyticus*
D1-13	BL	γ	-	+	*S.*spp
D1-13	FR	γ	-	-	S.spp
D1-14	FL	α	-	-	*S.chromogenes*
D1-17	FR	β	-	+	*S.haemolyticus*
D1-20	FL	β	+	+	*S. aureus*
D1-26	FR	γ	-	+	*S.chromogenes*
D1-43	BR	γ	+	-	*S.agnetis*
D1-43	FR	β	-	+	*S.haemolyticus*
D1-49	FL	β	-	-	*S.saprophyticus*
D1-49	FR	α	-	-	*S.xylosus*
D2-25	FL	β	-	-	*S.chromogenes*
D2-25	BL	β/α	-	-	*S.chromogenes*
D2-34	BL	β	-	+	*S.devrise*
D2-34	BR	β	-	+	*S.haemolyticus*
D2-37	FL	β	+	+	*S. aureus*
D2-37	FR	β/α	-	+	*S.haemolyticus*
D2-37	BL	β	-	+	*S.haemolyticus*
D2-37	BR	β	-	+	*S.haemolyticus*
D2-38	FL	β	-	-	*S.haemolyticus*
D2-38	BR	β	+	+	*S. aureus*
D2-41	FL	β	-	+	*S.haemolyticus*
D2-41	BR	β/γ	-	-	*S.haemolyticus*
D2-43	FL	β	-	+	*S. aureus*
D2-43	FR	α	-	+	*S.haemolyticus*
D2-43	BL	β	-	-	*S.haemolyticus*
D2-43	BR	β	-	+	*S.haemolyticus*
D2-44	FL	β	-	+	*S.haemolyticus*
D2-44	FR	β	-	-	*S.haemolyticus*
D2-44	BL	β/γ	-	+	*S.haemolyticus*
D2-47	BL	β	-	-	*S.haemolyticus*

[a] Hemolysis on MP2
[b] coagulase tube
[c] isdA PCR
[d] 16S rRNA sequence

Sequence Alignments and Predicted Tertiary Structure

The variable region of IsdA was predicted to comprise amino acids 222–308 (NCBI). Primers that encompass this region were used for *isd*A PCR and sequencing (GE Healthcare). Alignments and evolutionary analysis were completed in MEGA 7 [184]. A

rooted dendrogram of exhaustive pairwise IsdA alignments of 53 sequences (Table S2, Supporting Information) was constructed using maximum likelihood based on the JTT matrix-based model [184]. The I-TASSER server was used to visualize predicted IsdA tertiary structures [50]. Model templates included the *S. aureus* hemoglobin-IsdH complex and the NEAT domain of IsdA from *S. aureus* [185–188]. The Newbould 305 model has a C score of –1.93 (estimated TM score of 0.48 ± 0.15), and the MRSA252 model has a C score of –2.46 (estimated TM score of 0.43 ± 0.14). In the I-TASSER server, the range of C scores is –5 to 2, with >–1.5 indicating correct global topology.

Table S2. IsdA sequences for phylogenetic analysis

Strain	Host	Variant	Accession
Newbould 305	**cow**	**I**	**EJE55319**
RF122	cow	I	Q2YX95
LGA251	cow	II	CCC87810
112808A	cow	III	EOR47976
CVMN26035PS	chicken	I	KST23617
S54F9	pig	III	KPA00104
K12PJN53	pig	II	KII21536
SA7112	pig	III	KST55542
S123	pig	III	EPZ10793
ST772-MRSA-V	human	I	ABX29083
TCH130	human	I	253728336
TW20	human	I	CBI49003
JKD6008	human	I	ADL65130
ST228	human	I	CCJ22550
Newman	human	I	BAF67313
CN1	human	I	AGU61191
Z172	human	I	AGY89229
04-02981	human	I	ADC37297
A8796	human	I	EFH35863
CGS01	human	I	315196851
CIGC348	human	I	EHT89412
FCFHV36	human	I	AKA99128
Gv69	human	I	AIU85137
NRS100	human	I	AHW66048
MRGR3	human	I	EOR40242
MRSA S4	human	I	KSA20724
MRSN 2761	human	I	KKJ47858
TCH959	human	I	KLM30023
USFL270	human	I	CFE56532
DAR4145	human	I	AJE64530
MSSAT145	human	I	SCS53121
08-02300	human	I	ANI74018
6850	human	I	AGU54846
JKD6159	human	I	ADL22931

MO13	human	II	AEV78123
SA268	human	II	AII55584
ST1413	human	II	EWC67239
MRSA252	**human**	**III**	**CAG40105**
CIGC341D	human	III	EHT84578
CIG149	human	III	EHT78741
08BA02176	human	III	AFR73148
TCH60	human	III	ADQ77565
DR10	human	III	EIA14898
71193	human	III	AFH69349
S100	human	III	EPZ05864
VET0293R	human	III	EZS55766
ST20130939	human	I	AMV90129
CA-347	human	-	AGO29398
SK1585	human	-	KFD32297
UK2	human	III	WP054190280
S. aureus subsp. anaerobius	human	-	KKI67573
S.schwertzeri	human	-	CDR24069
S. argenteus	human	-	WP000160870

Anti-IsdA Mouse Serum

Mouse immunization protocols were pre-approved by the Boise State University IACUC. Immunizations were performed as described [161]). Briefly, two groups of six female BALB/c mice, 7 to 9 weeks old (Taconic, Hudson, NY), were administered 17 μg purified IsdA (MRSA252 variant III, pBA009) + 5 μg native cholera toxin (CT, Sigma Aldrich, St. Louis, MO, USA), or mock (PBS) in 10 μL 1XPBS applied to each nare by pipette under light anesthesia on days 0 and 10. Blood samples were obtained by lateral tail vein on day 14. Blood was allowed to coagulate and serum diluted 10-fold in protease inhibitor (as above). IsdA-specific antibodies in serum were analyzed by ELISA as described and endpoint titers defined as the reciprocal of the dilution giving an O.D. of 0.2 [65].

Adhesion Blocking and Ecm Binding Assays

For blocking assays, black walled (Nunc, Thermo-Fisher) 96-well plates were coated with 50 μL of 10 μg/mL bovine FN (R&D Systems, Minneapolis, MN, USA) for 12 h at 4◦C. A 1:100 dilution of heat inactivated pooled mouse serum was mixed with 1 ×

108 CFU of *S. aureus* MRSA252 or Newbould 305, grown in low iron media (LIM: 2 g NaCl, 1.2 g NaHCO3, 1.6 g yeast extract, 6 g protease peptone/400 mL), for 30 min at 37◦C. A portion of 100 μL of the mixture was added to plates coated with FN and incubated at 37◦C for 12 h prior to washing and addition of 150 μL Alamar Blue (0.1% reazzurin in 1× PBS). Plates were read at 530/590 nm after 24 h. For binding assays, IsdA from pBA009A and pCK001 was purified as above and separated on 12% SDS-PAGE. Purified IsdA (100 μg) was added in 2-fold dilutions to 96-well plates coated with 10 μg/mL bovine FN or bovine FG (Alfa Aerar, Reston, VA, USA) and incubated at 37◦C for 12 h prior to washing and addition of mouse anti-HIS (1:1000 Sigma) and anti-mouse HRP secondary (1:10 000 Promega). Plates were developed (TMB One) and read at 370 nm.

Graphing and Statistical Analysis

Graphing and statistical analysis was performed with *JMP* SAS software (Cary, NC, USA). PCR and RT-PCR are representative of three independent assays; ELISAs are reported as the means of three independent assays; and the OSP, adhesion and FN/FG binding assays are triplicate means of one assay that is representative of three independent assays. Significance for ELISA was determined using the non-parametric Wilcoxon rank score (Mann-Whitney) test. Significance of OSP and adhesion assays was determined using Student's *t*-test. *P*-values are reported as $P \leq 0.05(*)$, $P \leq 0.01(**)$ or $P \leq 0.0001(****)$.

Results

Presence and Expression Of isdA In Bovine Isolates of Staphylococci

Blood and milk were obtained from 103 cows at two US dairies that differed in size and operation. Presumptive staphylococci from milk were assayed by PCR for the presence of *isd*A and 16S rRNA species identification. Four isolates of 34 (12%) were *Staphylococcus aureus* and 19 were *isd*A positive (56%). Twenty-five additional clinical bovine *S. aureus* isolates were obtained. The presence of *isd*A, and six additional virulence factors, was assessed by PCR in all staphylococci (Table 2-2). *isd*A was conserved in *S. aureus* (total of 28/29, 96.6%), and also present in *S. haemolyticus* (11/17, 64.7%), *S. chromogenes* (2/5, 40%) and *S. devreise* (1/1, 100%). *isd*B was conserved in all *S. aureus* (29/29, 100%), and other species harboring *isd*A. The adhesin clumping factor A (*clf*A) was also highly conserved (28/29, 96.6%). To determine the expression of *isd*A during mammary infection, we identified the presence of *isd*A mRNA from total milk RNA. As shown in Fig. 2-1A, the majority of *isd*A positive staphylococci-infected milk samples (*Staphylococcus*+ *isd*A+) contained *isd*A mRNA. The staphylococcal culture from 14FL (D1) was negative for *isd*A by PCR, and the milk sample was negative by RT-PCR. Two *Staphylococcus* +*isd*A+ samples had low CFUs, and were inconclusive by RT-PCR (D1-26FR and D2-43FL). The expression of IsdA *in vivo* was assessed by anti- IsdA ELISA of milk and serum. As shown in Figure 2-1B and C, *Staphylococcus*+ *isd*A+ milk and serum samples had heightened anti-IsdA IgG responses over samples negative for *Staphylococcus* (*Staphylococcus*– *isdA*–). A western blot of purified IsdA probed with *Staphylococcus*+ *isd*A+ milk (cows D2 43FR and D2 37FR) compared to *Staphylococcus* – *isd*A – milk (cows D2 1FL and D2 6FL) supported

the immunogenicity of IsdA *in vivo* (Figure 2-1D). To assess the efficacy of anti-IsdA serum antibodies, we determined OSP of *S. aureus* Newbould 305 (Figure 2-1E). Bacterial cells were mixed with bovine PBMCs and *Staphylococcus–isd*A– serum (low anti- IsdA titer, light gray bars) or *Staphylococcus +isd*A+ serum (high anti-IsdA titer, dark gray bars). Serum was preincubated in the presence or absence of purified IsdA to block antibody activity. High anti-IsdA serum stimulated more bacterial killing than low anti-IsdA serum ($P \leq 0.05$), and purified IsdA significantly blocked bacterial killing in the presence of high anti-IsdA serum ($P \leq 000.1$).

Table 2-2 PCR analysis of adhesins from bovine staphylococcal isolates

Sequence ID	***# of total isolates***	**# positive isolates** *(percent positive)*						
		***isd*A**	***isd*B**	***isd*H**	***clf*A**	***fnbp*A**	***nuc***	***mec*A**
S. aureus	**29**	**28***(96.6)*	**29***(100)*	**25***(86.2)*	**28***(96.6)*	**25***(86.2)*	**26***(89.7)*	**0**
S. haemolyticus	**17**	**11***(64.7)*	**11***(64.7)*	**0**	**0**	**4***(23.5)*	**0**	**0**
S. chromogenes	**5**	**2***(40)*	**2***(40)*	**0**	**2***(40)*	**2***(40)*	**0**	**3***(60)*
S. agnetis	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
S. xylosus	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
S. devriesei	**1**	**1***(100)*	**1***(100)*	**0**	**1***(100)*	**0**	**0**	**0**
S. saprophyticus	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**1***(100)*
***S.* spp.**	**3**	**1** *(33.3)*	**1***(33.3)*	**0**	**0**	**0**	**0**	**1** *(33.3)*
Newbould 305		+	+	+	+	+	+	
USA300		+	+	+	+	+	+	+

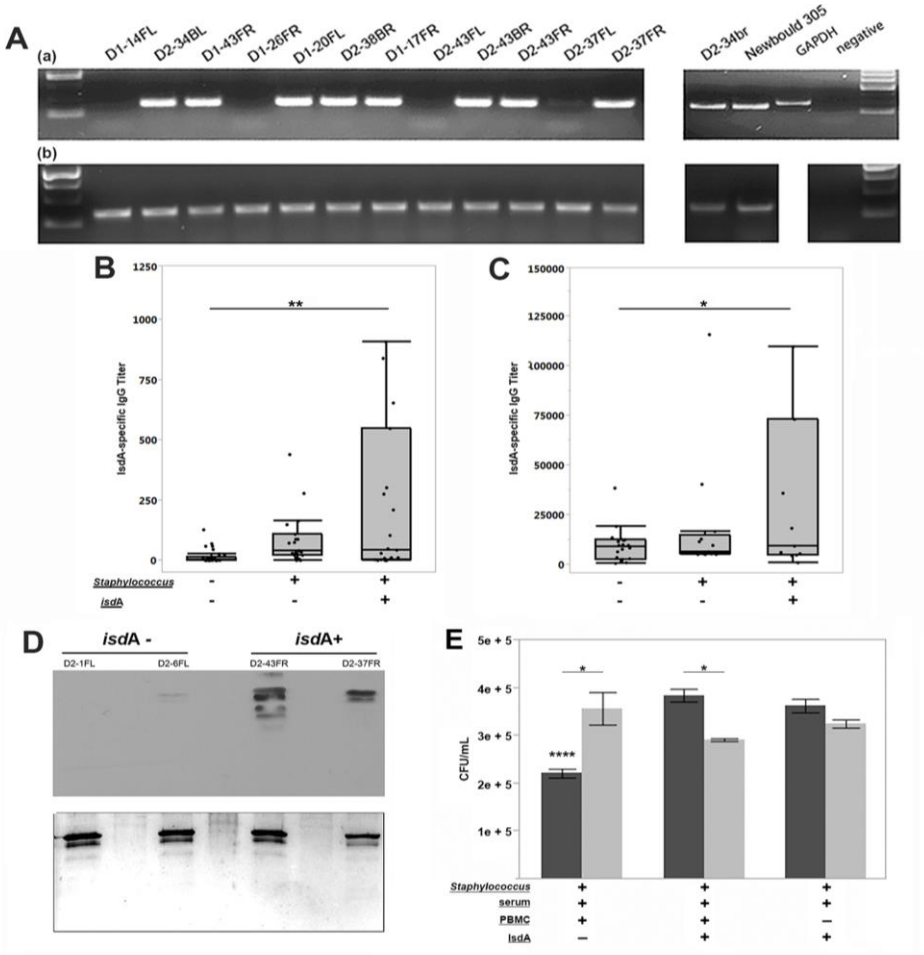

Figure 2-1. ***In vivo*** **expression and immunogenicity of Staphylococcus IsdA from bovine milk and serum. (A) Expression of isdA from total RNA in bovine milk: RT-PCR of (a) isdA (385 bps) and (b) 16S rRNA (530 bps). (B) IsdA-specific IgG responses in milk and (C) serum as determined by ELISA on samples obtained from Dairies 1 and 2. Samples are grouped as those from cows that were negative or positive by culture of Staphylococcus in milk and those that are negative or positive for isdA by PCR. Significance using the non-parametric Wilcoxon rank score test (Mann-Whitney), P ≤ 0.05(∗) or P ≤ 0.01(∗∗), between cow groups is shown. (D) Western blot (top) and corresponding SDS-PAGE (bottom) of anti-IsdA responses in milk. Purified IsdA (62 kD) was probed with Staphylococcus–isdA– (D2-1FL, D2-6Fl) and Staphylococcus +isdA+ (D2-43FR, D2-37FR) milk samples. (E) OPA of *S. aureus* Newbould 305 with PBMCs and serum from Staphylococcus –isdA– (light gray bars) and Staphylococccus +isdA+ (dark gray bars) cows. Significance using the pairwise Student's t-test, P ≤ 0.05(∗) between serum types or P ≤ 0.0001 (∗∗∗∗) between treatment groups, is indicated. (Adapted from Misra et al. (2017)** [184] **)**

IsdA sequence analysis

Comparison of published *S. aureus* IsdA indicates that 86 amino acids within the C-terminus are variable. This region is distinct from conserved iron-binding, heme-binding and NEAT domains of IsdA, as well as the C-terminal cell wall sortase signal (Fig. 2-2A). The 380-bp variable region from all *isd*A +staphylococcal isolates were amplified for sequencing and alignment. Figure 2-2 shows IsdA alignments from staphylococci of the two dairies (B) and 23 *S. aureus* clinical isolates (C). One *S. aureus* was negative for *isd*A by PCR and one was *isd*A+ but sequence could not be obtained. Nucleotide differences within this region resulted in two main allelic variants that align with bovine (Newbould 305, variant I) and human (MRSA252, variant III) IsdA. Notably, variant III contains a characteristic four-amino-acid insertion near the C-terminus. Predicted tertiary structure models were constructed using IsdA from Newbould 305 and MRSA252 (Fig. 2-2D) [50]. Models predict that the variable region of IsdA has a coiled secondary structure with a solvent accessibility of 3–7 (0 = buried residue, 9 = highly exposed). To determine if observed variation is consistent with human *S. aureus* IsdA, phylogenetic analysis was completed with 53 published sequences (Figure 2-3). Results included four cows, four pigs and one chicken isolate. Sequences were largely conserved; however, differences within the variable region resulted in clustering within two groups represented by MRSA252 (III, blue) and Newbould 305 (I, red). A smaller subgroup consisted of those that contained only the characteristic four-amino-acid C-terminal insertion (variant II, green). Two strains (CA-347 and SK1585) are closely related to variant II, but do not contain this C-terminal insertion.

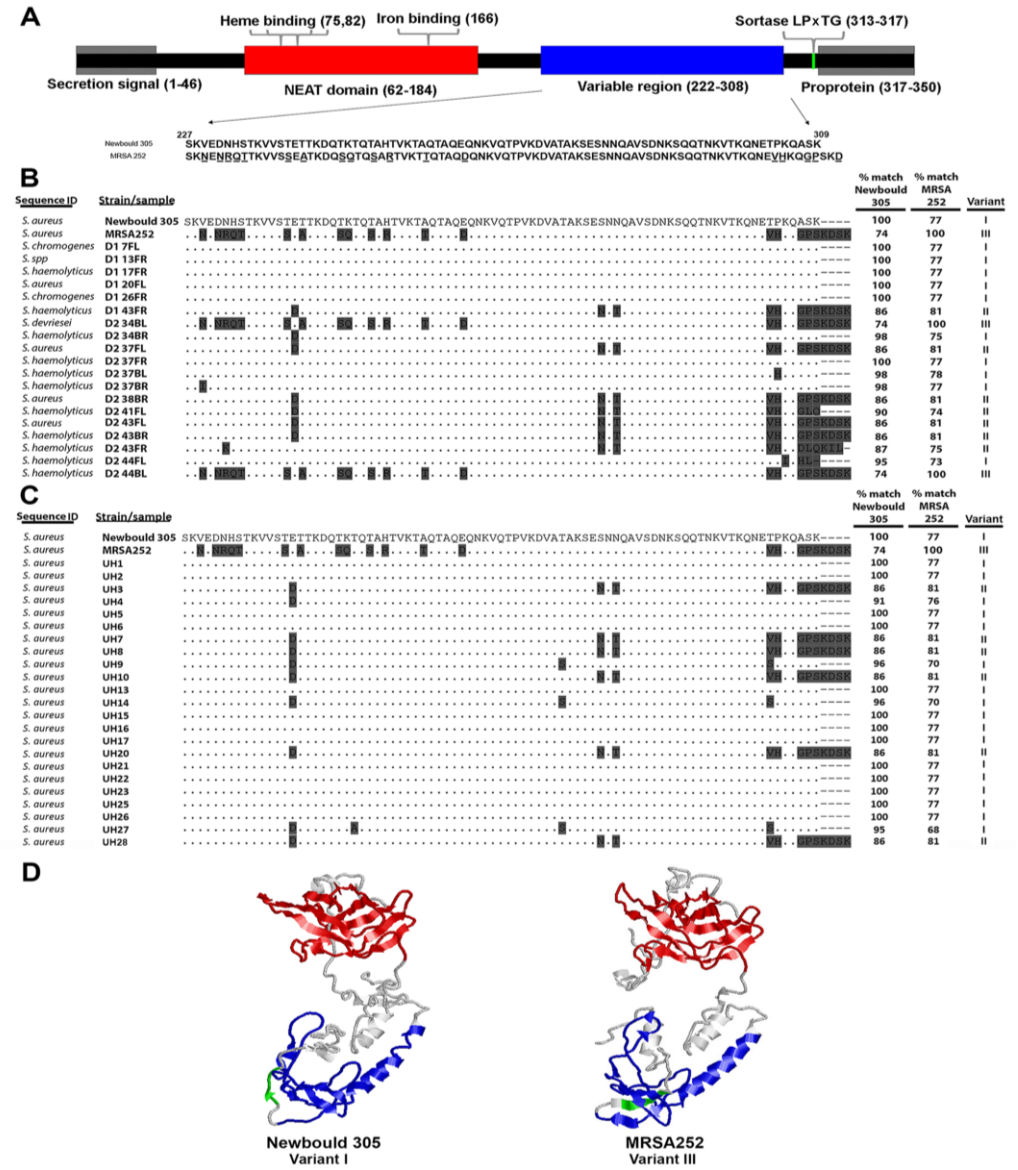

Figure 2-2. IsdA alignment and structure from bovine isolates. (A) Schematic representation of IsdA structure including the conserved NEAT domain (red), variable C-terminal regions (blue) and cell-wall-associated sortase signal (LPxTG, green). Predicted amino acid alignment of the IsdA variable region from (B) 19 isdA+ Staphylococcus from Dairies 1 and 2, and (C) 23 isdA + *S. aureus* from mastitic bovine milk across the USA, showing percent positive identity to *S. aureus* Newbould 305 and MRSA252. (D) I-TASSER predicted tertiary structures of IsdA from Newbould 305 (variant I) and MRSA252 (variant III) *S. aureus*. Colors are representative of the same regions as those in A. (Adapted from Misra et al. (2017) [184]**, thanks to Tyler Wines for sequencing and alignment)**

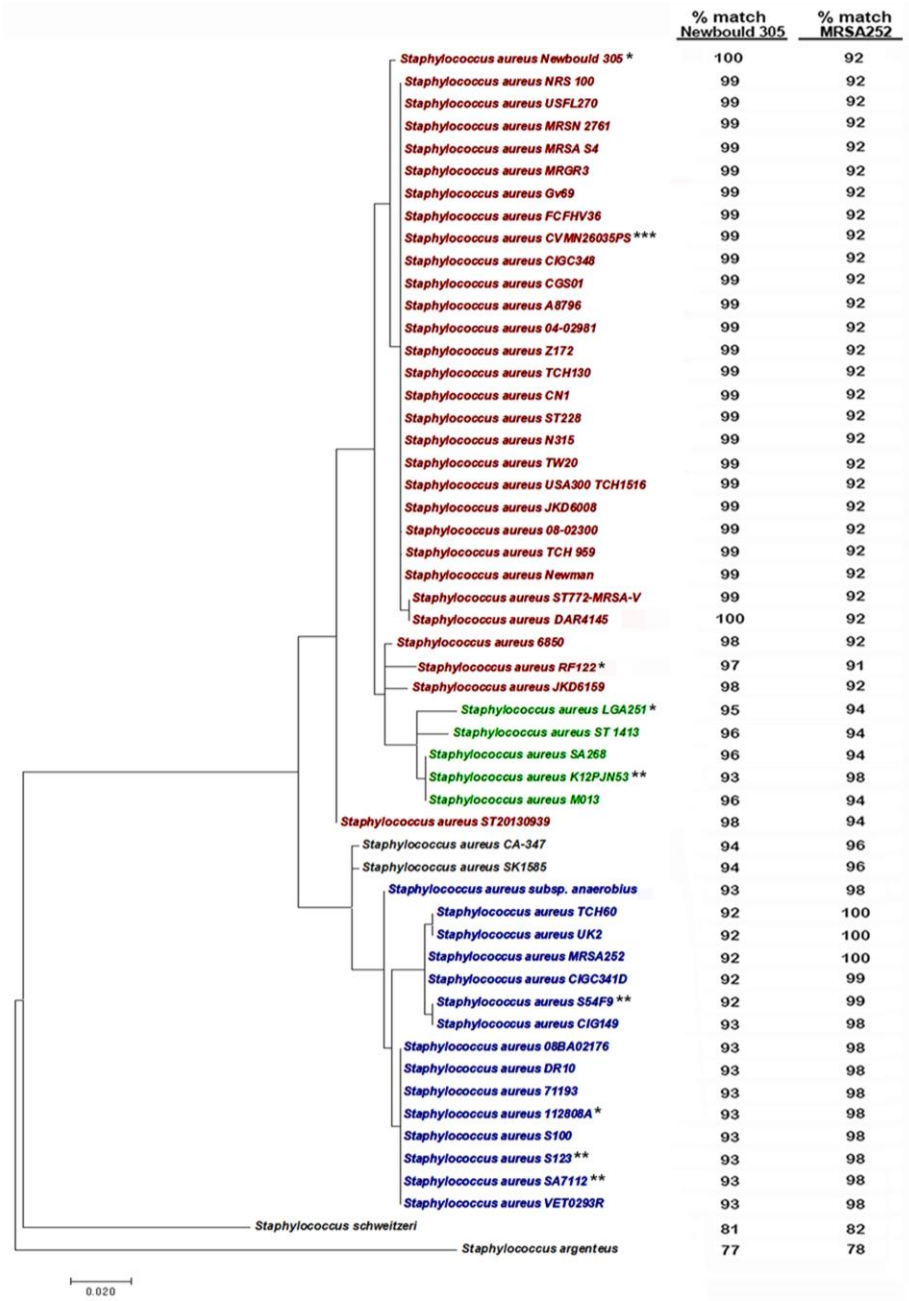

Figure 2-3. Dendrogram of pairwise IsdA alignments of 53 published sequences. Evolutionary analysis was performed with the maximum likelihood method based on the JTT matrix-based model (MEGA, Version 7). Variants align into three main groups, based on sequence differences within the variable C-terminus, representative of Newbould 305 (I, red), MRSA252 (III, blue) and LGA251 (II, green). Isolates from cow (*), pig (**) and chicken (***) are noted. (Adapted from Misra et al. (2017) [184])

Characterization of IsdA Variants

To assess if genetic variation of *isd*A could contribute to differences in protein immune reactivity, we produced antibodies to variant III (MRSA252) in mice (Figure 2-4A) and used pooled serum to block *S. aureus* adhesion to FN. Antibodies produced against variant III reduced FN binding of *S. aureus* MRSA252, but did not reduce binding of Newbould 305 (Fig. 2-4B). Variants I and III were purified from *Escherichia coli* and found to run at distinct sizes on SDS-PAGE (Figure 2-4C). ECM binding assays revealed that variant I (Newbould 305) and variant III (MRSA252) had consistently distinct interactions with FN, while variants bound similarly to FG (Figure 2-4D and E).

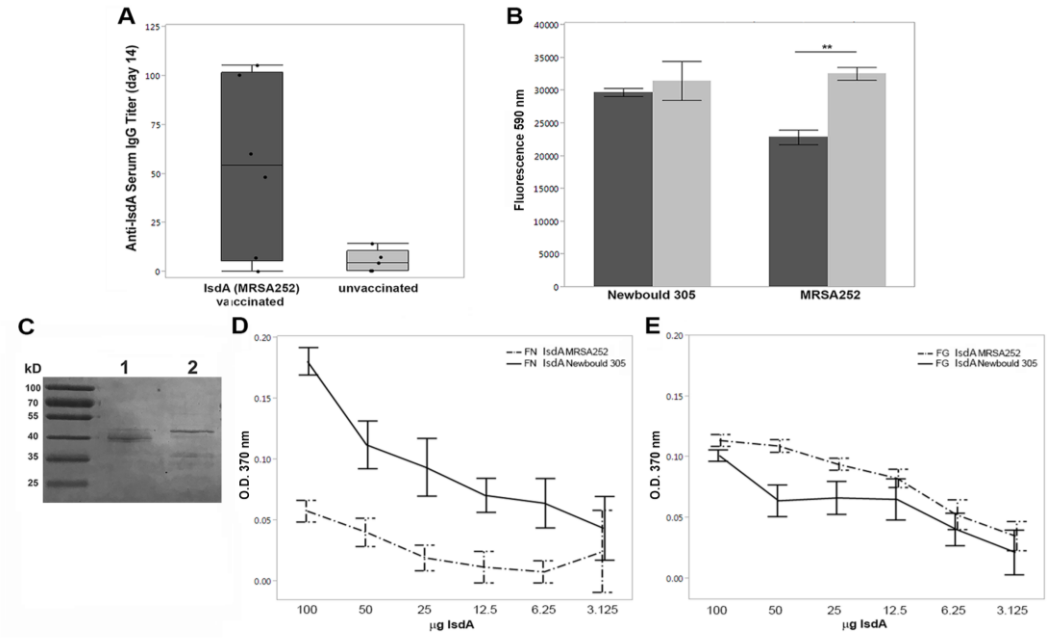

Figure 2-4. Characterization of IsdA variants. Immune reactivity of variants assessed by blocking *S. aureus* adhesion to FN in vitro with mouse antiserum. (A) IgG anti-IsdA titers from mice vaccinated with IsdA (variant III, MRSA252) or unvaccinated, and (B) adhesion of *S. aureus* Newbould 305 or *S. aureus* MRSA252 to FN after incubation with pooled mouse serum from mice vaccinated with IsdA MRSA252 (dark gray bars) or unvaccinated serum (light gray bars). Significance using Student's t-test, P ≤ 0.01(∗∗), between serum types is indicated. Binding specificity of IsdA variants assessed by ECM binding assay: (C) SDS-PAGE of purified IsdA from Newbould 305 (lane 1) and MRSA252 (lane 2), and binding of purified IsdA from MRSA252 and Newbould 305 to (D) FN and (E) FG. (Adapted from Misra et al. (2017) [184]**, thanks to Liwen Yang and Colton Knopp for mouse study and FN binding assays)**

Discussion

S. aureus is a prevalent pathogen in humans and livestock [8]. This bacterium has been investigated for decades, but its mechanisms of virulence are still not fully understood. This study was aimed at evaluating IsdA as a potential bovine vaccine candidate. The expression and variation of IsdA from a number of bovine *S. aureus* was analyzed to advance the current understanding of its involvement in bovine disease. The results from this study generated a number of outcomes and observations, including: *1)* the presence of *isd*A is strongly conserved in bovine *S. aureus* and it is also present in some species of coagulase negative (CNS) *Staphylococcus*, *2) isd*A is expressed in milk from infected cows and induces antigen-specific responses, *3)* anti-*isd*A antibodies are active to induce opsonophagocytosis, *4)* due to amino acid variation within the C-terminus, there are two-three main allelic variants of IsdA and *5)* allelic variants bind differentially to ECM proteins, indicating that the C-terminus is important for FN binding. Use of targeted and conserved antigenic proteins is an essential characteristic for a vaccine, especially for a highly variable pathogen like *S. aureus*, and this study advanced our understanding of one important antigen.

While the presence of *isd*A in CNS strains of *Staphylococcus* indicates that this antigen is not species-specific CNS staphylococci are also known to cause mastitis, and infections are clinically relevant and may become chromic [189]. Other antigenic surface proteins like ClfA and IsdB were also determined in this study to be conserved at the genetic level, however, further characterization is required to investigate their antigenicity. IsdB and ClfA both are reported potential vaccine antigens for human vaccines [47,190]. Previous reports show a non-significant stimulation of immune

responses by IsdB in human vaccines, however ClfA could be further characterized as an antigen for bovine vaccine [77]. In this study, significant stimulation of anti-IsdA immune responses in milk and serum was detected from cows currently infected with *isd*A+ staphylococci, reflecting effective immune reactivity and surface exposure of the IsdA antigen. The gene expression of *isd*A in milk also indicated that it is surface exposed at the time of infection, further supporting its use as a target antigen for bovine disease. However, a major limitation is of this study is that milk and blood samples were taken at one time point, and no information is available on how long cows were infected, or if cows had been infected previously. Thus, the efficacy, and the relevancy, of anti-IsdA antibodies was assessed by opsonophagocytosis assay (OPA). OPA using antibodies from serum of infected or uninfected cows shows stimulation of active immune responses against IsdA, which were able to uptake and trigger phagocytosis of *S. aureus.* These results indicate that IsdA is present and expressed from bovine *Staphylococcus in vivo* and that this antigen induces active immune responses *in vivo*.

The NEAT domain of IsdA has a role in binding specificities and immune reactivity, whereas the C-terminus is responsible for conferring hydrophobicity [54]. The hydrophobicity provided by the C-terminus is very important for skin colonization of *S. aureus* [54]. The results from multiple alignments in this study determined that there is variation at the C-terminus of IsdA that results in the occurrence of two main allelic variants. This variation is a result of amino acid insertions and a proline to histidine substitution within the C-terminus. These allelic variants were represented by two different strains of *S. aureus*; bovine (I, Newbould 305) and human (III, MRSA 252), with variant I actually predominating in *S. aureus* isolated from both hosts. The NEAT

domain of IsdA is known to bind to FG, and it is mostly conserved [62]. FN binding experiments in this study, revealed that variation at the C-terminal influenced the binding specificities of allelic variants, which suggests that the role of the C-terminus of IsdA is to aid in the binding of FN. The limited sample size of this report however does not support the conclusion that allelic variants have preference for either bovine or human hosts. Current evidence suggests that that both allelic variants of IsdA may be important for ECM interactions in bovine disease. Overall, the work presented here supports the inclusion of IsdA in a vaccine designed to combat *S. aureus* caused bovine mastitis, however, the incorporation of multiple alleles may be necessary.

CHAPTER THREE: IMMUNOGENICITY OF A MUCOSAL *STAPHYLOCOCCUS AUREUS* VACCINE FOR BOVINE MASTITIS

Abstract

Staphylococcus aureus causes a chronic, contagious disease of the udder, or mastitis, in dairy cows. This infection is often refractory to antibiotic treatment, and has a significant economic impact on milk production worldwide. An effective vaccine to prevent *S. aureus* mastitis would improve animal health, reduce antibiotic dependence and inform human vaccine approaches. The iron-regulated surface determinant A (IsdA) and clumping factor A (ClfA) are conserved *S. aureus* extracellular-matrix adhesins and target vaccine antigens. Here we report the results of two bovine immunogenicity trials using purified IsdA and ClfA-cholera toxin A_2/B chimeras (IsdA-CTA_2/B and ClfA-CTA_2/B). Cows were intranasally inoculated with IsdA-CTA_2/B + ClfA-CTA_2/B at dry off and followed for 70 days. Trial 1 utilized three groups with one or two booster doses at a total concentration of 600 or 900 μg. Trial 2 utilized two groups with one booster at a total concentration of 1200 μg. Humoral immune responses in serum, milk and nasal wash were examined by ELISA. Responses in serum were significant between groups and provide evidence of antigen-specific IgG induction after vaccination in both trials. Cellular proliferation was detected by flow cytometry using antigen-stimulated PBMCs from day 60 of Trial 2 and revealed an increase in CD4+ T cells from vaccinated cows. IsdA and ClfA stimulation induced Il-4 expression, but not IFN-γ or IL-17, in PBMCs from day 60 as determined by cytokine expression analysis. Opsonophagocytosis of *S.*

aureus confirmed the functional *in vitro* activity of anti-IsdA antibodies from Trial 2 serum and milk. The vaccine was well tolerated and safe, and results support the potential of mucosally-delivered CTA_2/B chimeras to protect cows from mastitis caused by *S. aureus*.

Introduction

Bovine mastitis is one of the most prevalent and economically impactful diseases of the dairy industry. The economic losses caused by this disease are estimated to be $1.7-2 billion annually in the U.S. [14,15]. Multiple pathogens are capable of causing bovine mastitis, leading to loss of milk yield and deteriorating animal health, however, *S. aureus* causes contagious and difficult or impossible to treat subclinical and clinical bovine mastitis [191,192]. *S. aureus* caused bovine mastitis is also of great concern due to increasing antibiotic resistance and overall decrease in cure rates, especially for β-lactamase positive isolates [193]. It has been reported that approximately 4-10% of cows in U.S. are infected by *S. aureus* [174,194–196]. Management control practices on farms have been successful in decreasing the rate of infection, however, *S. aureus* is known to colonize skin and internalize inside mammary epithelial cells, alveolar cells, and macrophages, which can limit antibiotic efficacy and lead to chronic, difficult to treat subclinical infection [99]. An effective bovine *S. aureus* vaccine to reduce or eliminate disease and prevent transmission would have large impacts on both animal and human health [197–200].

There have been a number of attempts to design an *S. aureus* vaccine for bovine mastitis. Approaches like whole cell vaccines, live attenuated vaccines, DNA vaccines, toxoids, capsular polysaccharides and purified proteins have been used [129,201–204].

Despite substantial progress, an effective vaccine that can protect cows against the multiple strains and serotypes of *S. aureus* that cause mastitis is still needed. It is well recognized that the use of multiple highly conserved and significant virulence factors will be necessary to induce inter-strain protection. Additionally, the route of vaccine administration and use of adjuvant for a mucosal and intracellular pathogen like *S. aureus* is critical. A vaccine delivered mucosally, that could induce mucosal humoral as well as cellular responses would reduce the colonization and adherence of *S. aureus* and aid in preventing chronic *S. aureus* mastitis.

In this study, two bovine immunogenicity trials were performed to assess the activity of a subunit vaccine comprised of purified iron regulated surface determinant protein A (IsdA) and clumping factor A (ClfA) proteins combined with cholera toxin (CTA_2/B) adjuvant (IsdA-ACT_2/B +ClfA-CTA_2/B). CTA_2/B is a safe and immunogenic mucosal adjuvant from *Vibrio cholerae* made by genetically removing the toxic CTA1 subunit [161,163,205]. CTA_2/B is known to stimulate cellular and protective immune responses when co-administered with antigens [162–168, 203, 204].

We hypothesized that a CTA_2/B adjuvant based mucosal vaccine would induce antigen-specific humoral and cellular immunity, and will aid in reducing colonization of *S. aureus* in the cow udder. Our results support the conclusion that intranasal administration of the IsdA-CTA_2/B+ClfA-CTA_2/B subunit vaccine during the dry period can stimulate significant antigen-specific immune responses in milk and serum and that the antibodies are active and capable of triggering opsonophagocytosis.

Materials and Methods

Bacterial Strains, Growth Conditions and Plasmids

Staphylococcus aureus Newbould 305 was used for the cloning of *isd*A and *clf*A and opsonophagocytosis assay (OPA)[206–208]. *S. aureus* was grown Luria Broth (LB) with shaking at 37°C for genomic DNA isolation and in low iron media (LIM) without shaking at 37°C for OPA [33]. *E. coli* TE1 was used for cloning and protein expression of HIS-tagged antigens from pCK001 and pMaH001 for ELISA (Table 3-1). These strains were grown in LB for cloning, or terrific broth (TB) for protein expression, + 100 μg/mL ampicillin at 37°C. *E. coli* Clear Coli BL21(DE3) (Lucigen, Madison, WI) was used for expression of CTA_2/B chimeras from pLR001 and pLR003 and grown at 37°C in TB in presence of 35 ug/mL chloramphenicol. Plasmid pLR001 was constructed by amplification of Newbould 305 genomic DNA using *isd*A-specific primers and ligation into the vector pARLDR19 (SphI-ClaI) [169]. pLR003 was similarly constructed by cloning into pARLDR19 (SphI-XhoI) using *clf*A-specific primers. pCK001 was constructed by ligation of *isd*A from Newbould 305 into pTRCHIS (BamHI-HindIII) to express HIS-IsdA. pMaH001 was constructed by cloning of Newbould 305 *clf*A, using pLR003 as a template, into pBAD18 (NheI-HindIII) to enable expression of ClfA-HIS into the *E. coli* periplasm. Strains, plasmid details and primer sequences (with restriction sites underlined) are shown in Table 3-1. All plasmids were sequenced through cloning junctions for confirmation.

Table 3-1. Bacterial strains, plasmids and primers used in this study

Bacterial strains		
	Genotype or characteristics	**Reference or source**
E.coli TE1	Δ*endA* derivative of TX1	[79] [79]
E.coli ClearColi®	Lipid IV_A modification of BL21(DE3)	Lucigen, Madison, WI
S. aureus Newbould 305	Bovine clinical isolate	[32]

Plasmids			
	Gene	**Vector**	**Reference or source**
pLR001	*isd*A (Newbould)	pARLDR19	This study
pLR003	*clfA* (Newbould)	pARLDR19	This study
pCK001	*isd*A (Newbould)	pTRCHisA	This study
pMAH001	*clf*A (Newbould)	pBAD18	This study

Primers			
	Gene	**Amplicon**	**Source**
Cloning			
pLR001: **FW** GCTACTGGCATGCGGCAACAGAAGCTACGAAC **RV** GTGCATGATCGATTTTGGTAATTCTTTAGC	*isd*A	846 bp	[21]
pLR003: **FW** GACTGGGCATGCAGGTGTAACTTCAACTGCTAAAG **RV** GCATGGCTCGAGGGAATTGGTTCAATTTCACCAG	*clf*A	840 bp	This study
pCK001: **FW** GCTACTGGATCCGCGGCAACAGAAGCTACGAAC **RV** GTGCATAAGCTTTCAAGTTTTTGGTAATTCTTTAGC	*isd*A	852 bp	[21]
pMAH001: **FW** GATCCTCGCTAGCGGATGGAGTAAGACCATGG **RV** GTACCAGAAGCTTTTAGTGATGGTGATGGT GATGGGGAATTGGTTCAATTTCAC	*clf*A	994 bp	This study
Bovine cytokine qPCR:			
F**W** GCATCGTGGAGGGACTTATGA **RV** GGGCCATCCACAGTCTTCTG	GAPDH		[80]
FW CAGCATGGAGCTGCCT **RV** ACAGAACAGGTCTTGCTTGC	IL-4		[81]
FW CAGAAAGCGGAAGAGAAGTCAGA **RV** TGCAGGCAGGAGGACCAT	INF-γ		[80]
FW GCCCACCTACTGAGGACAAG **RV** GCTGGATGGTGACAGAGTTC	IL-17A		[82]

Protein expression and purification.

Chimeras were purified as described [209]. Briefly, to express IsdA-CTA_2/B and ClfA-CTA_2/B, ClearColi® (Lucingen, Madison, WI) transformed with pLR001 or pLR003 was grown to an optical density (O.D.600) of 0.9 and induced for 24 h with 0.2 % L-arabinose. Proteins were isolated from the periplasmic extract with 1 mg/mL polymyxin B and purified by affinity chromatography on immobilized D-galactose

(Pierce™ D-Galactose Agarose, Thermo Fisher, Waltham, MA). Vaccine proteins were dialyzed into sterile 20% glycerol + 1XPBS and protein concentrations determined by BCA (Pierce™ BCA, Thermo Fisher). Vaccines were tested to ensure endotoxin levels were below 0.05 EU/mL (LAL Endpoint Chromogenic, Lonza, Allendale, NJ) plated for sterility and stored at -80°C until use. For use in ELISA, IsdA and ClfA from Newbould 305 were isolated from the cytosol of *E. coli* Top10 (Thermo Fisher) containing pCK001 and pMAH001 after overnight induction with 1M IPTG. Proteins were then purified on cobalt (HisPur™, Thermo Fisher) under denaturing conditions and dialyzed into sterile 1xPBS. For use in flow cytometry and OPA, endotoxin was removed from these proteins (Pierce™ Endotoxin Removal Columns, Thermo Fisher).

Animals, Trial Design and Sample Collection

All animal protocols were pre-approved by the Boise State University Institutional Animal Care and Use Committee. Approval was obtained from the State of Idaho and USDA Center for Veterinary Biologics to transport exploratory vaccine. Sample size was determined prior to Trial 1 by power analysis based upon predicted immune responses in milk and serum. A sample size of 7 cows per group was predicted to provide, at a 95% level of confidence, 92% power to detect a minimum difference between the control and any other group of 15%. This was based on a univariate two-group repeated measures analysis of variance (ANOVA), assuming that the between groups error term is 0.13 and the within-groups error term is 0.08 [210]. Healthy Holstein cows in the third or fourth lactation were enrolled in the study (21 total Trial 1, n =7; 22 total Trial 2, n=11). Animals were initially screened as those with at least one of two previous somatic cell counts (SCC) counts below 200 $x10^3$ cells/mL and no clinical

evidence of mastitis. Further enrollment criteria included: *1)* no growth of *S. aureus* culture from milk, *2)* low baseline anti-IsdA responses in milk, and *3)* no evidence of bovine leukemia virus (BLV) infection. Cows were randomized into two or three groups (Figure 3-2) and vaccinated on days 1, 14 and 49 (Trial 1) or 1 and 14 (Trial 2), and followed until calving (Day Fresh = day 60 or 70). One cow in Trial 1 (Group 2) and four cows in Trial 2 (Groups 1 and 2) left the study prior to day 60 due to unrelated complications. Milk was not obtained from two additional cows in Trial 1 (Groups 1 and 3) because they did not freshen by day 70. The vaccine was delivered in 2.5 mL volumes into each nare using a nasal cannula (Merck & Co., Kenilworth, NJ). Blood was isolated from tail vein and allowed to coagulate at room temperature (RT) for 1hr prior to centrifugation and resuspension into 1:10 Inhibitor Solution (IS =1X HALT ™ protease inhibitor, and 5% glycerol in 1X PBS), or collected as whole blood in vacutainer tubes for peripheral blood mononuclear cell (PBMC) isolation (Becton Dickinson, Franklin Lakes, NJ). Composite milk samples were taken aseptically after washing teat ends with 70% ethanol. Milk was centrifuged at 700 xg for 20 min at 4°C for removal of fat, and skim milk was stored in 1:10 IS at -20°C prior to use in ELISA. Nasal antibodies were obtained by insertion of absorbant cotton into each nare and resuspension of cotton into 5 mL of 1:10 prior to freezing at -20°C and use in ELISA. Nasal cultures were taken by insertion of cotton swabs into each nare (Hygiena Q-swab™, Thermo Fisher).

Milk Culture, PCR and Clinical Assessment

100 μL and 10 μL of milk and nasal culture were plated separately on MP2 (Udder Health Systems, Inc., Meridian, ID). The presence of small, white, esculin-negative colonies was considered presumptive staphylococcal species. These colonies

were isolated and genomic DNA analyzed by PCR for confirmation of staphylococcal species (primers, Chapter 2, Table 2-1), as well as the *S. aureus*-specific gene *nuc*, and *isd*A and *clf*A. All animals were scored for the presence of clinical mastitis by temperature and observation of udder on days of vaccination and milk sampling [211,212]. SCC analysis was performed on milk using the California Mastitis Test (CMT, Udder Health Systems, Inc., Meridian, ID).

IgG and IgA ELISA

IsdA and ClfA specific immune responses on serum, milk and nasal wash were detected using ELISA, as described [161]. Briefly, 96-well microtiter plates (Nunc, Thermo Fisher) were coated with 10 μg of IsdA or ClfA in 50μL 1XPBS, blocked for 2 hr at 37°C in 1% goat milk + 1X PBS, and incubated with two-fold dilutions of bovine serum, purified milk IgG or nasal wash from Trials 1 and 2. Dilutions were initiated at a 1:10 concentration of serum, and a 1:2 concentration of nasal wash for Trial 1, and for Trial 2 the total IgG concentration was determined and concentrations of serum were initiated at 50 μg. Purified milk IgG was obtained using protein G affinity (Nab^{TM} Protein G and Zeba^{TM} Spin, Thermo Fischer) and dilutions for ELISA were initiated at 25 μg concentrations for both trials. Plates with antibody dilutions were incubated at 4°C for 12 hr prior to washing and addition of HRP-anti-bovine IgG (1:10,000 Bethyl Laboratories, Montgomery, TX) or HRP-anti-bovine IgA (1:10,000 Bethyl Laboratories, Montgomery, TX). Samples were developed with tetramethylbenzidine (Promega^{TM} TMB One, Thermo Fisher) and read at 370 nm. Serum and milk ELISAs were performed on individual cows for Trial 1 (n=6-7), or pools of 6-7 cows by vaccine group for nasal wash. For Trial 2, ELISA results are reported using pools of 1-2 cows (n=5) for consistency with cellular

analysis. Data are presented as the ratio of Day Fresh (or Day X for serum)/Day1 of the optical density (O.D. 370 nm) from a representative antibody dilution in the linear part of the curve. Results are the average of three independent assays.

PBMC Isolation, Flow Cytometry and Cytokine qPCR

PBMCs were isolated from whole bovine blood from Trial 2 on Day 60. PBMCs were isolated using a density gradient established by layering whole blood diluted 1:2 with PBS on Histopaque®1077 (Sigma-Aldrich, St. Louis, MO). Blood samples were centrifuged at 800 ×g for 30 min at room temperature. The buffy coat was removed and washed 3 X with Hank's buffered salt solution (HBSS) for 10 min at 400 ×g at room temperature (RT) and counted with 0.2% Trypan blue. Mononuclear cells (PBMCs) were then pooled such that each pool contained cells from 2 cows in each pool (*n*=4-5). PBMCs (1×10^5) were plated in tissue-culture treated 96-well microtiter plates (Corning, Sigma-Aldrich) and grown in RPMI-1640 at 37 °C, 5% CO_2. Cells were stimulated with 10 µg of IsdA + ClfA endotoxin free antigen on days 1 and 3, and harvested on day 6. Cells were then stained with 10 µL/10^6 cells anti-CD4 FITC (CC8, BioRad, Hercules, CA) and anti-CD8 PE antibodies (CC63, BioRad), or equivalent amounts of isotype control (IgG2a FITC, Becton Dickenson), for 30 min in the dark at 4 °C. Samples were washed with 1xPBS, centrifuged at 400×g for 5 min, and resuspended in 3% FBS/0.02% NaN_3/PBS. CountBright™ absolute counting beads (10 µL) (Thermo Scientific) were added to determine absolute cell numbers. Samples were analyzed for fluorescence intensity on a FACS Calibur flow cytometer (Becton Dickinson) and FlowJo data analysis software (Tree Star, Inc.). A minimum of 10,000 total lymphocyte events were collected for each reaction. For cytokine assays, total RNA from day 6 of

antigen stimulated pooled PBMCs (*n*=4-5) was extracted (RNeasy, Qiagen, Germantown, MD) with an additional DNase I (Promega, Madison, WI) digestion. cDNA was reverse transcribed as per manufacturer (High-Capacity RNA-to-cDNA™ Kit, Thermo Fisher). qRT PCR was conducted using SYBR fast (Kapa Biosystems, Thermo Fisher) on interferon gamma (IFN- γ), interleukin-4 (IL-4) and interleukin-17 (IL-17) primers, using bovine GAPDH as reference gene (Table 3-1). The data are presented as fold changes determined by stimulated vs non-stimulated PBMC. All qRT-PCR experiments were performed twice using biological triplicates.

Opsonophagocytic Assay (OPA)

OPA was performed as described [68,213]. Briefly, equal concentrations of endotoxin free IsdA antigen (50 ug/well), and pooled (*n*=5) heat inactivated serum (50ug/well) or purified milk IgG antibodies (50ug/well) from days 20 and 60/Fresh of Trial 2 were mixed and incubated for at 37°C, 5% CO_2 for 1 hour. After incubation, equal volumes of *S. aureus* Newbould 305 ($2x10^5$ CFU) and bovine PBMCs ($2x10^5$ cells) were added, and the reaction was further incubated for 90 minutes. PBMCs were isolated as described above from whole citrated bovine blood (Hardy Diagnostics, Santa Maria, CA). After 90 min, 10 µl samples of reaction mixture were plated on MP2 in triplicate at 1:100,000 to determine CFU/mL. Results are reported as the percent killing, or the reduction in CFUs/ml with serum/milk IgG compared to PBMC's and *S. aureus* alone without serum/ milk IgG.

Statistical Methods and Analysis

Serum ELISA results from both trials were analyzed using repeated measures ANOVA on logged values in a mixed-model framework with compound symmetric

within-cow covariance. Vaccine groups were explicitly compared on each day with hypotheses not adjusted due to pre-planned comparisons. A Wilcoxon test in an exact testing framework was used to compare vaccine treatments for Trial 2 flow cytometry and qPCR data. Serum percent kill from OPA was analyzed as 3-way ANOVA (with and without IsdA, vaccine group, and day) and within this model vaccine group was compared within day and IsdA status, and IsdA status was compared within vaccine status. Due to the nature and number of comparisons, sets of tests were adjusted for multiple comparisons using the false discovery rate method [214]. Milk percent kill from OPA was analyzed similarly to the serum, but in a 2-way ANOVA as only milk from day of freshening was used. Logged humoral responses in the nasal wash were analyzed with a 2-way ANOVA, with vaccine groups compared within each day (not adjusted due to pre-planned comparisons). Logged values were used where necessary to stabilize variance in residual plots, and nonparametric exact methods were used with the smallest sample sizes and where logging did not correct the skew of the data. Statistical analyses were conducted using JMP (Cary, NC) and SAS software (Cary, NC). *P*-values are reported as $p \leq 0.05$(*), $p \leq 0.01$(**) or $p \leq 0.0001$(****) and all reflect two-sided tests.

Results

Expression and Purification of IsdA-CTA$_2$/B and ClfA-CTA$_2$/B

CT is an enterotoxin from *V. cholerae* which acts as a stimulatory and delivery mucosal adjuvant. The crystal structure of CT reveals an AB_5 structure with a toxic A subunit and non-toxic pentameric B subunit (Figure 3-1A) [133]. To construct a mucosal subunit *S. aureus* vaccine, the toxic CTA_1 domain (*ctx*A) was genetically replaced by *isd*A or *clf*A, in the parental vector pARLDR19 (Table 3-1), to form a holotoxin-like

CTA_2/B chimera (pLR001 and pLR003, Figure 3-1B and D). IsdA-CTA_2/B and ClfA-CTA_2/B chimeras were expressed in *E.coli* using the leader sequence LTIIB of *E.coli* to direct the secretion of IsdA-CTA_2, ClfA-CTA_2 and monomeric CTB to the periplasmic space [29]. The folded subunits were purified from the periplasm using D-galactose agarose affinity chromatography. The binding of CTB to the column and co-purification with the CTA_2 fusion indicates the formation of chimeric holotoxin. The expressed chimeras were dialyzed, quantified and confirmed by SDS-PAGE (Figure 3-1C and 1E). IsdA-CTA_2 (≅38 kD) and ClfA-CTA_2 (≅37 kD) were co-purified with CTB (11kD). IsdA-HIS and ClfA-HIS were also purified from plasmids pCK001 and pMaH001 (Table 3-1). Expression was quantified and confirmed by SDS-PAGE for the use of these proteins in ELISA.

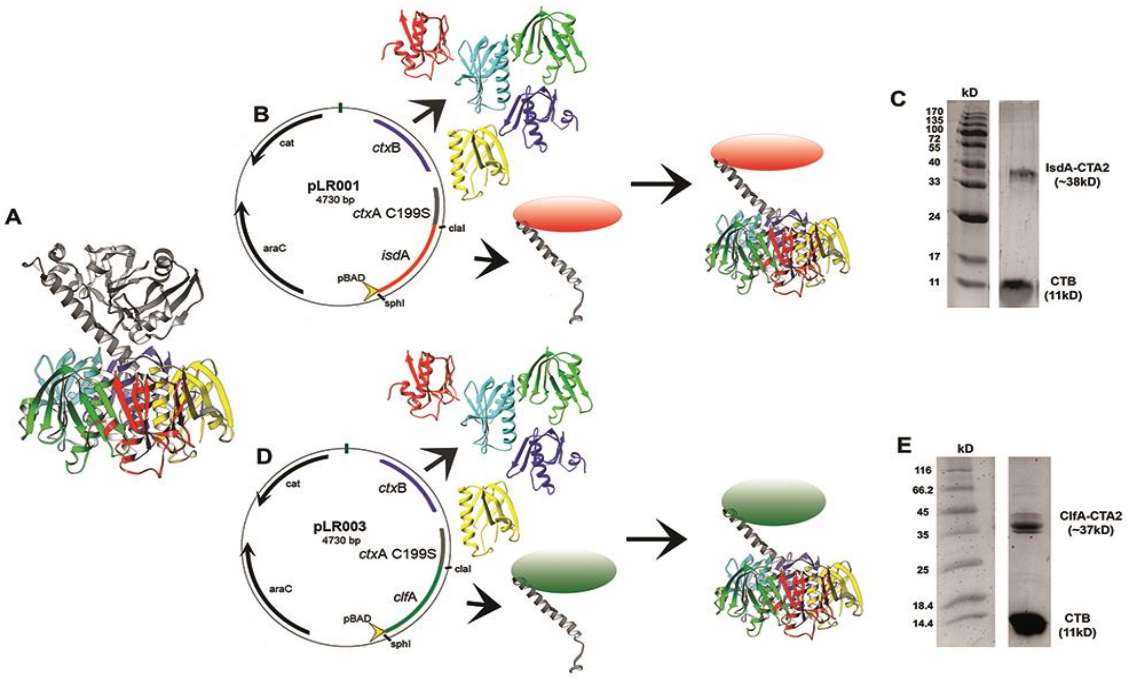

Figure 3-1. Construction and purification of *S. aureus* CTA$_2$/B chimeric mucosal vaccines. (A) ribbon diagram of *Vibrio cholerae* cholera toxin [38], (B) expression of the IsdA-CTA$_2$/B chimera from *E. coli* + pLR001, (C) SDS-PAGE of D-galactose affinity-purified IsdA-CTA$_2$/B (IsdA-CTA$_2$ = 38kD, CTB = 11kD) vaccine preparation, (D) expression of the ClfA-CTA$_2$/B chimera from *E. coli* + pLR003, and (E) SDS-PAGE of D-galactose affinity-purified ClfA-CTA$_2$/B (ClfA-CTA$_2$ = 37kD, CTB =11kD) vaccine preparation.

Trial Design, Safety Assessment and Culture Analysis

Two immunogenicity field trials were conducted in healthy Holstein cows during the dry-off period and shortly after parturition. Figure 3-2 shows the summary of trial design. Trial 1 group 1 (red) received 300 µg intranasal doses of IsdA-CTA$_2$/B + ClfA-CTA$_2$/B on days 1 and 14, group 2 (grey) was vaccinated on days 1, 14 and 49, and group 3 (light blue) received only mock (PBS) vaccination. Trial 2 group 1 (green) received 600 µg intranasal doses of IsdA-CTA$_2$/B + ClfA-CTA$_2$/B on days 1 and 14 and group 2 (dark blue) received PBS on these days. Animals were assessed during both trials for changes in temperature, SCC and clinical outcome (Table 3-2), and milk/nasal wash samples were

cultured for the presence of *Staphylococcus* and genotyping of isolates (Table 3-3). No animals showed evidence of inflammation or clinical mastitis during either trial, and temperatures, as well as SCC, were not affected by vaccination. No animals in either trial were *S. aureus* positive in milk or nasal wash at any time. The presence of all *Staphylococcus*, and those specifically harboring *isd*A and *clf*A, was monitored throughout each trial by culture and PCR. These efforts revealed that vaccination had no effect on the presence and numbers of *Staphylococcus* in general. In addition, while a small number of animals were found to harbor *isd*A and *clf*A positive *Staphylococcus* prior to vaccination (4 total, Trial 2, both groups, determined to be *S. hemolyticus and S. chromogenes*), no *isd*A/*clf*A positive staphylococci was present after vaccination in any group in either trial.

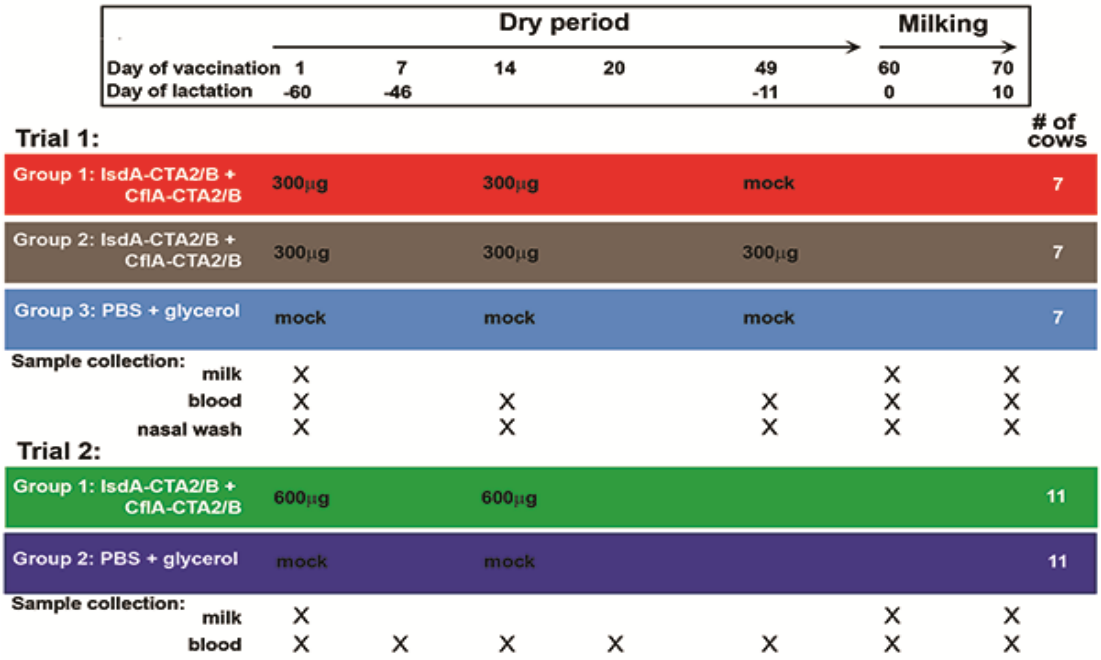

Figure 3-2. Trial 1 and Trial 2 summary showing vaccination schedule, dosage, sample collection and number of animals Groups are: 1) Trial 1, Group 1, vaccinated 2X on days 1 and 14 (red); 2) Trial 1, Group 2, vaccinated 3X on days 1, 14 and 49 (grey); 3) Trial 1, Group 3, not vaccinated (light blue); 4) Trial 2, Group 1, vaccinated 2X on days 1 and 14 (green); and 5) Trial 2, Group 2, not vaccinated (dark blue).

Table 3-2. Temperature and somatic cell count (SCC) from Trial 1 and Trial 2.

	Trial	*Group*	**Day**											
			Screen[c]	1	2	3	14	15	16	49	50	51	60	70
Temp[a]	**1**	1	-	**101.16**	**101.10**	**100.40**	**101.10**	**100.61**	**100.51**	**101.21**	**100.60**	**100.69**	**101.31**	**101.34**
		2	-	**100.93**	**101.71**	**100.61**	**101.46**	**100.54**	**100.59**	**101.56**	**100.66**	**100.52**	**101.70**	**101.77**
		3	-	**101.09**	**101.64**	**99.89**	**101.23**	**100.24**	**100.61**	**101.13**	**100.71**	**100.66**	**101.57**	**101.06**
	2	1	-	**100.82**	**98.50**	**98.75**	**99.35**	-	-	-	-	-	-	-
		2	-	**101.05**	**98.30**	**98.58**	**99.46**	-	-	-	-	-	-	-
SCC[b]	**1**	1	**49.86**	-	-	-	-	-	-	-	-	-	**296**	**851**
		2	**155.25**	-	-	-	-	-	-	-	-	-	**847.29**	**52.33**
		3	**150.86**	-	-	-	-	-	-	-	-	-	**126.71**	**132.14**
	2	1	**68.27**	-	-	-	-	-	-	-	-	-	**588.50**	**71.50**
		2	**210.55**	-	-	-	-	-	-	-	-	-	**1079.8**	**68.50**

[a] temperature in Fahrenheit by rectal thermometer
[b] SCC in cells/mL X1000 as determined by California Mastitis Test (CMT)
[c] sample taken on day of screening; -14 for Trial 1 and -9 for Trial 2.

Table 3-3. Culture and genotyping of Staphylococcus

PCR primers	**Sequence**	**Gene**	**Amplicon**	**Reference**
	Staphylococcus: **FW** GGCCGTGTTGAACGTGGTCAAATCA **RV** TIACCATTTCAGTACCTTCTGGTAA	*tstaG*	370 bp	[83]
	S. aureus: **FW** GCGATTGATGGTGATACGGTT **RV** AGCCAAGCCTTGACGAACTAAAGC	*nuc*	270 bp	[84]
	*isd*A: **FW** CGGTTCAACCAAAACCTGCT **RV** GCGAAGGCAACTGTGCTAAT	*isdA*	380 bp	[68]
	*clf*A: **FW** CCTATGCCAGT**A**GCCAATGTC **RV** GCACCAAGCAGGTTATGTC	*clfA*	318 bp	[68]

Percent Staph positive[a]	**Trial**	**Sample**	**Group**	**Day**					
				Screen[b]	1	14	49	60	70
	1	nose	1	**-**	**0**	**57**	**14**	**14**	**29**
			2	-	**29**	**57**	**0**	**50**	**17**
			3	**-**	**0**	**14**	**29**	**43**	**43**
		milk	1	**14**	**-**	**-**	**-**	**-**	**0**
			2	**29**	**-**	**-**	**-**	**-**	**17**
			3	**29**	**-**	**-**	**-**	**-**	**14**
	2	milk	1	**9**	**-**	**-**	**-**	**-**	**22**
			2	**27**	**-**	**-**	**-**	**-**	**40**

[a] percent of cows in each vaccine group positive for staphylococcal species as determined by culture and PCR.
[b] Groups are defined as: Trial 1: 1=vaccinated 2X, 2=vaccinated 3X, 3=mock; and Trial 2: 1= vaccinated 2X, 2=mock.

IsdA and ClfA-Specific Humoral Responses in Trials 1 and 2

Antigen-specific humoral responses were quantified by ELISA from blood, milk and nasal wash collected during Trial 1, and from blood and milk collected during Trial 2. Results are reported as a ratio of Day X/Day 1 for serum and nasal wash, and Day Fresh/Day 1 for milk. In Trial 1, group 2 (grey bars, Figure 3-3A), induced significantly higher IsdA-specific IgG responses in serum on days 60 and 70 (p = 0.027 and 0.047, respectively), with a consistent increase in vaccine group 1 (red bars), over unvaccinated group 3 (light blue bars). Anti-IsdA IgG responses from group 2 on day 49 also indicated an increase relative to unvaccinated group 3 (p = 0.080). Anti-ClfA responses were supportive of an increase in ClfA specific IgG responses from groups 1 and 2 on days 60 and 70, but not significant (Figure 3-3B). Similarly, IsdA specific immune responses in milk after freshening were the highest in vaccine group 2, followed by vaccine group 1,

relative to unvaccinated group 3, but not significant (Figure 3-3C). Anti-ClfA responses in milk were more variable, but with a lower ratio in the unvaccinated group compared to both vaccinated groups (Figure 3-3D). In Trial 2, the vaccinated group 1 (green bars, Figure 3-4A) showed a significant increase in the IsdA-specific IgG immune responses over unvaccinated group 2 (dark blue bars) in serum on days 20 and 49 (p = 0.009 and 0.0003, respectively), and a supportive increase on day 60. Anti-ClfA responses in serum were consistent with anti-IsdA responses, but were significant on day 20 only (p = 0.013) (Figure 3-4B). IsdA-specific IgG responses in milk upon freshening were also higher for the vaccinated group in this trial, but not significant (Figure 3- 4C). Lastly, the ClfA antigen did not stimulate detectable IgG immune responses in milk over unvaccinated (Figure 3-4D). Nasal wash anti-IsdA IgA was also assessed from pooled samples obtained in Trial 1, and results are supportive of a stimulation of antigen-specific IgA, especially within group 2 (Figure 3-5 grey bars, group 2 showing higher response than group 3 on day 70 only, p = 0.043). Together, results demonstrate that antigen-specific humoral responses can be stimulated after intranasal vaccination with IsdA-CTA_2/B + ClfA-CTA_2/B.

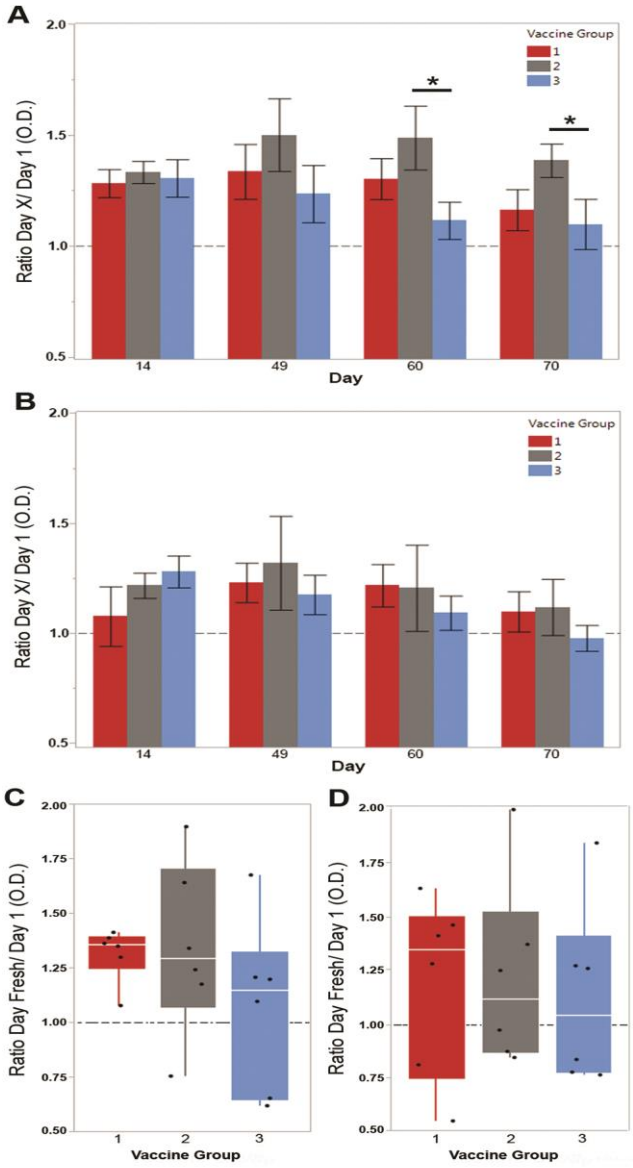

Figure 3-3. Trial 1 humoral responses in serum and milk. Anti- IsdA (A) and anti-ClfA (B) IgG responses as determined by ELISA using serum from days 14, 49, 60 and 70. Results are reported as ratios of Day X/Day (O.D.) at serum dilution of 1:320. Anti-IsdA (C) and anti-ClfA (D) IgG responses as determined by ELISA using IgG purified from milk on day 60 or 70 (Day Fresh). Results are reported as ratios of Day Fresh/Day 1 (O.D.) at IgG dilution of 1:80 (3.125 μg). Groups are: 1) vaccinated 2X on days 1 and 14 (red), 2) vaccinated 3X on days 1, 14 and 49 (grey), and 3) not vaccinated (light blue). Data are presented as the mean ± standard error for serum (n=6-7), or quartile box plots with medians for milk (n=6). Significance (*) on Day 60 and Day 70 (p = 0.027 and p = 0.047) is shown.

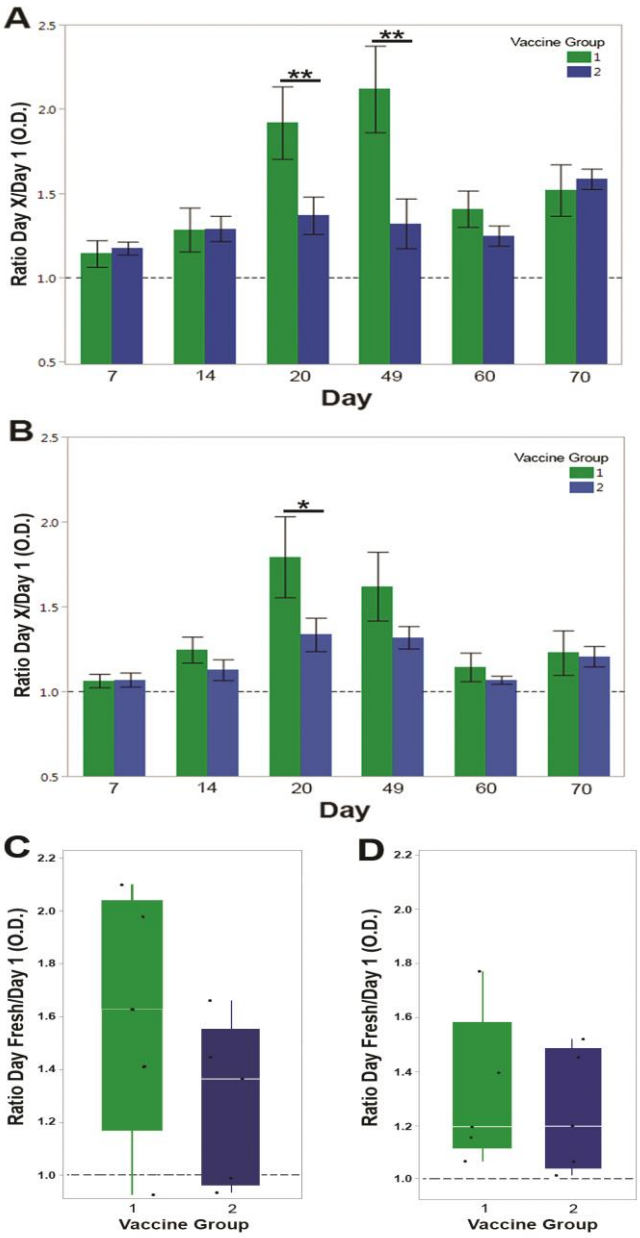

Figure 3-4. Trial 2 humoral responses in serum and milk. Anti- IsdA (A) and anti-ClfA (B) IgG responses as determined by ELISA using serum from days 7, 14, 20, 49, 60 and 70. Results are reported as ratios of Day X/Day 1 (O.D.) at serum dilutions of 1:1280 (400 ng total protein). Anti-IsdA (C) and anti-ClfA (D) IgG responses as determined by ELISA using IgG purified from milk on day 60 or 70 (Day Fresh). Results are reported as ratios Day Fresh/Day 1 (O.D.) at milk dilution of 1:80 (3.125 μg). Groups are: 1) vaccinated 2X on days 1 and 14 (green), 2) not vaccinated (dark blue). Data are presented as the mean ± standard error for serum or quartile box plots with medians for milk (n=5). Significance (**) on Days 20 and 49 for anti-IsdA responses (p = 0.009 and p = 0.0003) and (*) on Day 20 for anti-ClfA response (p = 0.013) is shown.

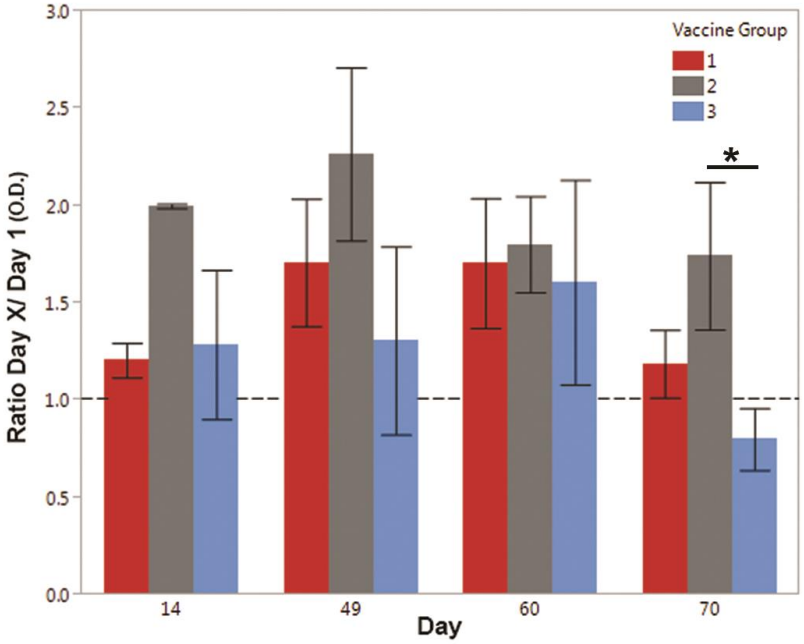

Figure 3-5. Trial 1 humoral responses in nasal wash. Anti-IsdA IgA responses in pooled nasal wash on days 14, 49, 60 and 70. Results are reported as ratios of Day X/Day1 (O.D.) at a nasal wash dilution of 1:16. Groups are:1) vaccinated 2X on days 1 and 14 (red), 2) vaccinated 3X on days 1, 14 and 49 (grey) and 3) not vaccinated (light blue). Data are presented mean ± standard error (n=3); significant difference between vaccine groups 2 and 3 on day 70 (*, *p* = 0.043) is shown.

Induction of Cellular Immune Responses from Trial 2

The stimulation of T cell responses was assessed after the isolation of PBMCs from cows on day 60 of Trial 2, and the stimulation of these cells *in vitro* with purified IsdA+ ClfA antigen. Flow cytometry was used to determine concentrations of CD8+ and CD4+ cells in stimulated and unstimulated groups (Figure 3-6A). Cows vaccinated with IsdA-CTA_2/B and ClfA-CTA_2/B on day 60, show a significant increase in the proliferation of CD4+ T cells after stimulation compared to the non-vaccinated control group (p = 0.032; Figure 3-6B). The vaccinated group also revealed a modest increase in the proliferation of CD8+ T cells. The levels of IFN-γ, IL-4 and IL-17 cytokine

expression was also assessed by qRT-PCR from antigen (IsdA + ClfA) stimulated PBMCs on day 60. PBMCs from cows immunized with IsdA-CTA_2/B + ClfA-CTA_2/B revealed significantly higher expression levels of IL-4 upon stimulation ($p = 0.016$), but no detectable increase in INF-γ or IL-17 (Figure 3-6C). These results indicate that the mucosal immunization with IsdA-CTA_2/B and ClfA-CTA_2/B stimulated a Th2-type cellular response.

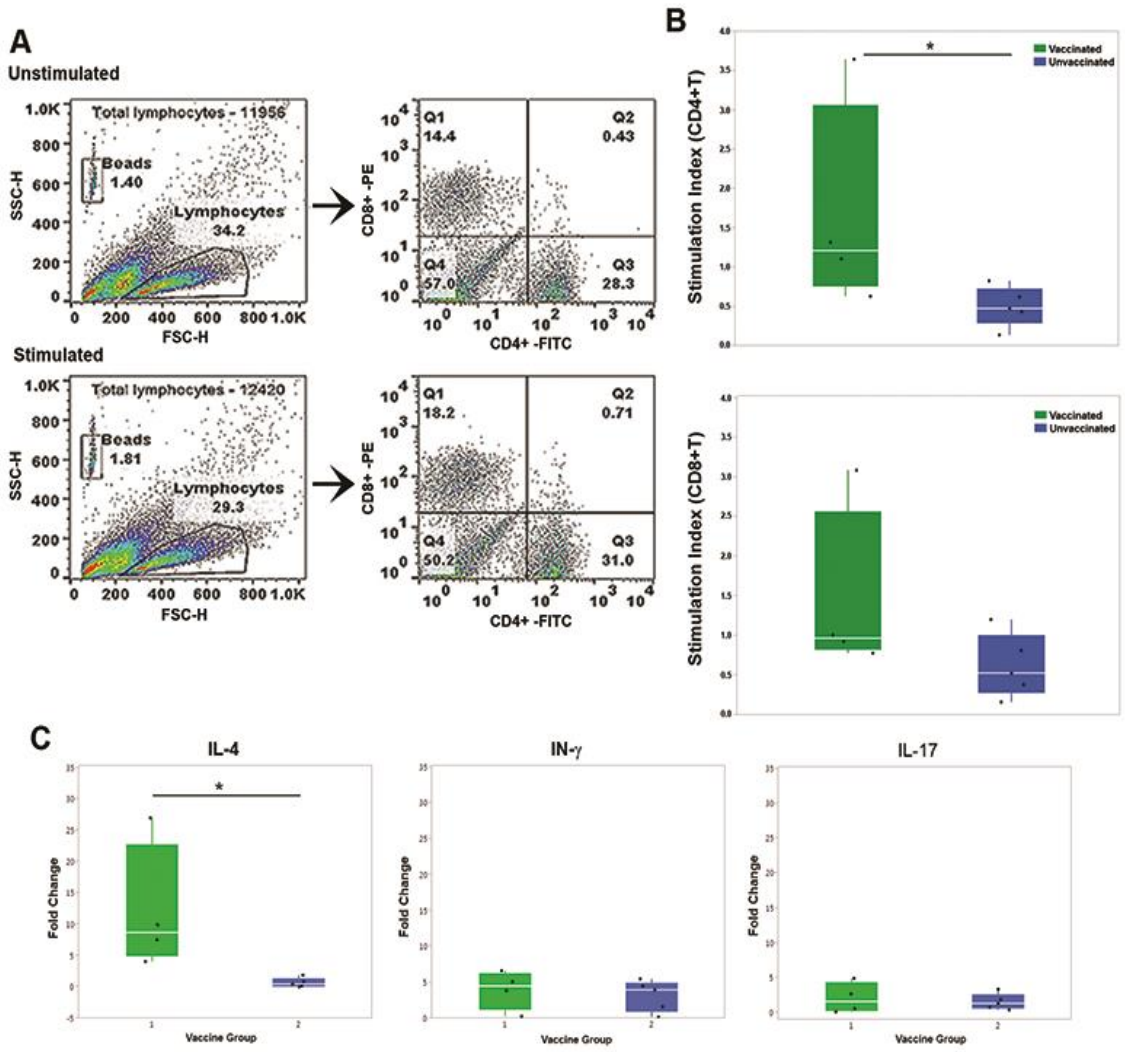

Figure 3-6. Trial 2 cellular and cytokine responses. Flow cytometric analysis to determine proliferation of CD4+ and CD8+ T-lymphocytes from Day 60 after stimulation with IsdA + ClfA: (A) representative light scatter dot plots showing the gated bead and lymphocyte populations for unstimulated and stimulated PBMCs, as well as the lymphocyte gated population of CD8-PE vs CD4-FITC, where the CD8 population falls in quadrant 3 (Q3) and CD4-FITC population in quadrant Q4, and (B) quantification of flow cytometry showing the CD4+ T cell and CD8+ T cell stimulation index in vaccinated (green) and unvaccinated (blue) groups. Significance (*) for CD4+T cell stimulation is. (C) Quantitative RT-PCR to determine cytokine expression from Day 60 antigen-stimulated PBMCs isolated from vaccinated (green) and unvaccinated (dark blue) cows, showing: IL-4, INF-γ and IL-17 expression. Data is reported as normalized Ct values fold change over GAPDH. Significance (*) for CD4+ T cell (p = 0.032) and IL-4 expression (p = 0.016) is shown.

Opsonophagocytic Activity of Isda Specific IgG Antibodies from Trial 2

The *in vitro* activity of antibodies stimulated against IsdA was assessed by OPA using serum and milk from days 20, 60 and fresh of Trial 2 (Figure 3-7). Serum from days 20 and 60 of vaccinated cows showed a significant increase in killing activity in the absence of purified IsdA ($p < 0.0001$ and $p = 0.009$, respectively). When purified IsdA was added to the assay, percent killing was reduced in the vaccinated group on both days ($p < 0.0001$ and $p = 0.001$ for days 20 and 60, respectively) (Figure 3-7A and B). Purified IgG from the milk of vaccinated cows upon freshening also showed significant opsonophagocytosis activity in the vaccinated group relative to the unvaccinated group ($p = 0.041$), and consistent blocking of killing activity in the presence of IsdA antigen in the vaccinated group ($p = 0.010$) (Figure 3-6C). Overall results of this assay indicate that, antibodies stimulated against IsdA are active against isogenic *S. aureus in vitro* and induce significant opsonophagocytic responses.

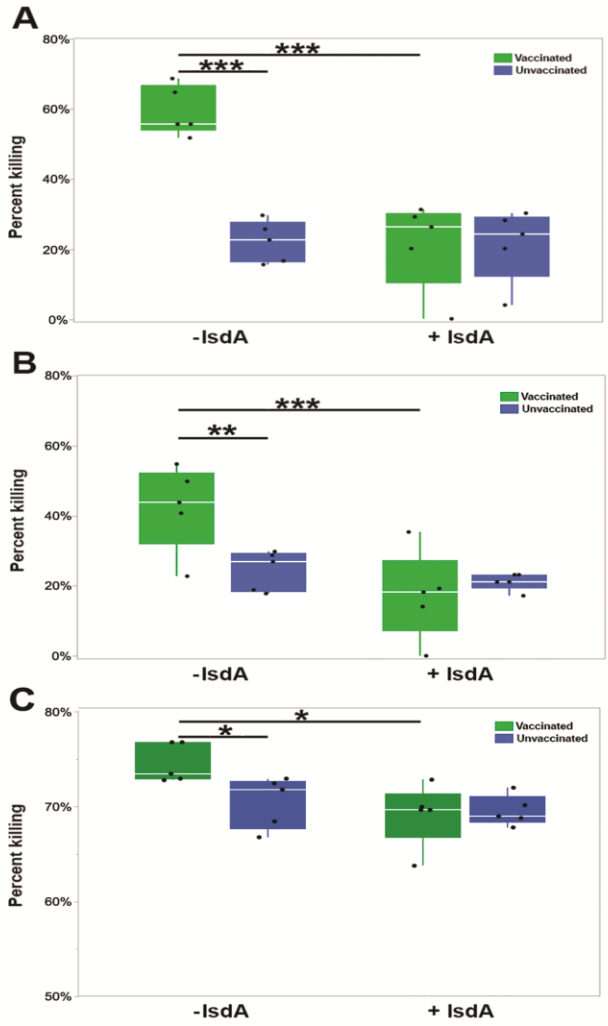

Figure 3-7. In vitro activity of anti-IsdA responses from Trial 2 serum and milk. Opsonophagocytic assay (OSP) to determine the ability of anti-IsdA humoral responses from days 20 (A) and 60 (B), or pooled milk from day Fresh (C), of vaccinated (green) and unvaccinated (dark blue) cows, to promote phagocytic killing. Samples were also pre-mixed with (+IsdA) or without (-IsdA) purified IsdA to assess the inhibition of serum activity. Results are reported as percent killing (CFU/mL) of *S. aureus* Newbould 305 by bovine PBMCs after mixture with pooled serum compared to a control of PBMCs plus *S. aureus* in the absence of serum (n=5). P-values are adjusted for false discovery rate [214]**. Significance between serum in vaccine groups without IsdA on days 20 and 60 is shown (p = < 0.0001 *** and p = 0.009 ** respectively); as is significance between IsdA presence and absence on days 20 and 60 (***, p < 0.0001 both days); in milk, vaccine groups differ (p = 0.041) and in the vaccinated group, percent killing differs by IsdA status (p = 0.01,* shown).**

Discussion

Bovine mastitis is one of the most important diseases of the dairy industry and developing methods of prevention is a top priority. New approaches that can induce complex responses to block colonization and transmission are needed. In this study, we describe the results of two clinical trials using a mucosal subunit vaccine (IsdA-CTA_2/B + ClfA-CTA_2/B) against *S. aureus* caused bovine mastitis. Both trials were conducted at the start of the lactation dry period, vaccine was administered intranasally and boosted either one or two times during the trial. Blood and milk samples were collected during and at the end of the trial to detect immune responses. Significant antigen-specific immune responses were detected in the serum of vaccinated animals in both the trials. In Trial 2, cytokine expression profile revealed that vaccine induced immune responses were largely Th2-type. Importantly, the antibodies that were stimulated were specific to IsdA, active and effective in triggering opsonophagocytosis *in vitro*. The IsdA-CTA_2/B + ClfA-CTA_2/B vaccine was found to be safe and well tolerated intranasally, at dosage as high as 1200 µg.

IsdA is a FN and FG binding surface adhesin from *S. aureus,* it also contributes to iron sequestration, surface hydrophobicity and lactoferrin resistance. Multiple reports have described IsdA as a central human antigenic vaccine target [62,64,161]. ClfA is an FN adhesin, also known to be conserved and immunogenic [124,195,209]. As described in Chapter 1, ECM binding is critical for colonization of *S. aureus* in the cow udder, as this interaction potentiates its uptake intracellularly into various cells like, macrophages, neutrophils, mammary gland epithelial cells (MacTs) [215,216].

Our results from both the trails indicated that there is an antigen-specific increase in humoral immune responses in vaccinated animals. Both IsdA and ClfA stimulated

immune responses, however, immune responses stimulated against IsdA were consistently higher in both trials, as compared to ClfA. This may be due to the structure and differential stability of IsdA-CTA_2/B compared to ClfA-CTA_2/B. The antigenic surfaces exposed during folding of chimera could facilitate higher antibody stimulation with more specificity for IsdA [217,218]. Also, antigens combined with CTA_2/B chimeras can potentially lose their tertiary structure, which is a limitation that can influence antibody stimulation and specificity [219]. The results from OPA indicated that anti-IsdA antibodies were induced and were responsible for the majority of the *in vitro* killing activity of *S. aureus*. These results indicate that, while anti-IsdA responses were induced to a higher level, addition of ClfA was beneficial, and this antigen may have contributed to cellular responses. Future incorporation of additional antigens will be essential to optimize efficacy.

Successful prevention by vaccine relies on the timing of vaccination and booster doses thereafter. In Trial 1, there were two vaccinated groups and one unvaccinated. The group that was vaccinated twice (1) was compared to group 2 vaccinated three times and the unvaccinated group. There was a slight but supportive booster effect seen after the third dose of vaccination in group 2 In Trial 2, the vaccination was administered at a higher dose, which induced higher immune responses in serum over the 50-day period of vaccination with a more distinct booster effect, however these responses declined sharply during the freshening period. Despite this drop, anti-IsdA IgG antibodies in milk were consistently elevated in vaccinated animals upon freshening. Importantly, milk antibodies from this trial, in which cows had received the last booster on day 14, were active and effective in OPA despite having lower serum responses. Opsonophagocytosis, or the demonstration of functional antibody activity, has been described as critical for a successful *S. aureus*

vaccine [220]. Here we revealed that the opsonophagocytic antibodies stimulated from both serum and milk during freshening were highly significant, antigen-specific, and supportive of their protective capacity.

Another key feature required for the success of a subunit vaccine is an adjuvant. Adjuvants are important for targeted delivery, antigen presentation and stability of the vaccine. CT, is a gold standard mucosal vaccine adjuvant that has been used extensively in animal studies [221–223]. CT has been reported to have anti-inflammatory properties [224,225]. CTA_2/B is a genetically engineered CT chimera that was first described as a mechanism to make stable human vaccines with the antigen directly coupled to a functional CTB subunit [226–229]. CTA_2/B chimeras have advantages over CT for use as mucosal vaccines, including: the absence of the toxic A_1 domain, the non-covalent association of antigen and adjuvant, and a native receptor-binding subunit that retains the A_2 ER-targeting KDEL motif [136]. Evidence suggests that an A_2/B chimeric structure may promote antigen uptake and presentation, resulting in efficacy in animal models [170,171,230]. Our results support the use of CTA_2/B chimeras for bovine vaccination, and suggest that intranasal vaccination with CTA_2/B can induce antigen-specific antibody production at the mucosal surface and in milk.

Despite decades of research, *S. aureus* has proven to be a difficult pathogen to eliminate. The development of an effective vaccine to prevent bovine mastitis caused by *S. aureus* would reduce bovine morbidity and mortality, increase food production and quality, reduce antibiotic use and potentially inform human vaccine development. This report describes the novel approach that may address these needs. Results are supportive of the continued assessment of CTA_2/B chimeras as bovine vaccines to prevent *S. aureus* mastitis.

CHAPTER FOUR: IMMUNOPROTEOMICS TO IDENTIFY *STAPHYLOCOCCUS AUREUS* ANTIGENS EXPRESSED IN BOVINE MILK DURING MASTITIS

Abstract

Staphylococcus aureus is an opportunistic pathogen affecting both human and animal species. An effective vaccine to prevent *S. aureus* bovine disease and transmission would have positive impacts on animal well-being, food production and human health. The objective of this study was to identify multiple immunogenic antigens that are involved in udder colonization and disease for incorporation into a vaccine to prevent bovine mastitis. *S. aureus* produces a number of cell wall anchored and surface-associated virulence factors that play key roles in the pathogenesis of mastitis. Many of these proteins are conserved between different strains and serotypes of *S. aureus*, and represent promising vaccine candidates. In this study, we utilized an immunoproteomics approach to identify antigenic proteins from the surface of *S. aureus*. The expression of cell wall and surface proteins from *S. aureus* was induced under low iron conditions, followed by trypsin extraction and separation by two-dimensional electrophoresis (2DE). The separated proteins were blotted with antibodies from mastitic bovine milk, and identified by liquid chromatography-mass spectrometry (LC/MS/MS) analysis. Thirty-eight unique proteins were identified, of which eight were previously reported to be involved in *S. aureus* virulence. Two immunogenic surface proteins; IsdC and EsxA, were cloned, expressed, and purified from *E.coli* for confirmation of immune reactivity by ELISA.

Amino acid alignments of published *S. aureus* sequences indicated that IsdC and EsxA are highly conserved. PCR on 37 bovine *S. aureus* isolates determined that the presence of *esx*A and *isd*C is also conserved. The immunoproteomics technique used in this study generated reproducible results and identified at least two promising vaccine antigens for use in novel vaccines to prevent bovine mastitis.

Introduction

Bovine mastitis is an important and economically relevant disease that affects the dairy industry worldwide [175,231]. This disease causes large economic losses every year; estimated to be as high as $2 billion/year in the U.S. alone [15,232]. These losses are mainly due to reduced milk yield, antibiotic therapy, veterinary services and the reduced price of culled animals [178,233]. Mastitis is most often an acute or chronic inflammation of mammary glands caused by bacterial infection, and commonly leads to increased somatic cell count (SCC), cytokine production, antibody secretion and bacterial load in milk [105,234]. Multiple pathogens are known to cause bovine mastitis, however, *S. aureus* is one of the most commonly isolated pathogens in milk, and an estimated 3% of dairy cows worldwide are infected [235]. *S. aureus* infection often results in chronic, subclinical disease that is highly contagious, and difficult or impossible to treat, with cure rates lower than 25% [236] . Despite decades of research, an effective vaccine that can prevent *S. aureus* bovine disease is not yet available; largely due to strain to strain variability and redundancy of *S. aureus* virulence factors. The strategy to design effective vaccines for *S. aureus* mastitis must include multiple conserved and immunogenic virulence factors that can provide cross-protection for the many strains of this bacterium.

S. aureus expresses a broad range of virulence factors, that include surface proteins covalently attached to the cell wall and secreted proteins expressed during infection [237]. These exposed proteins are essential for the survival and proliferation of *S. aureus*, and the presence of many of them is conserved [238,239]. Depending upon the stage of infection and physiological conditions inside the host, virulence factor expression will promote binding to the extracellular matrix (ECM), colonization, invasion, and avoidance of the immune response [240]. A number of these factors have been emphasized in both human and veterinary vaccines as important targets effective for vaccine development [201]. The iron-regulated surface protein A (IsdA) is an *S. aureus* surface adhesin that is also involved in iron sequestration and found to be highly expressed, conserved and immunogenic during in bovine mastitis [184]. Other adhesins, such as: IsdB, ClfA and HlA, also have antigenic properties in models for human *S. aureus* infection, however, their role in bovine disease requires further exploration [68,241].

Proteomics is an important tool to identify potential vaccine antigens, especially for pathogens like *S. aureus* with numerous surface exposed proteins [242,243]. Studies have reported the use of two-dimensional electrophoresis (2DE) for whole or subcellular proteome analysis of *S. aureus* for human vaccine development [244]. Couto *et* al. (2016), recently described the use of the SERPA (serome proteome analysis) technique to explore and characterize novel vaccine and therapeutic targets for *S. pseudointermedius* infections in dogs [245]. Mastitic cow milk specifically contains neutrophils, lymphocytes and antibodies as a result of infection and subsequent inflammation [92]. Immune cells, secreted antibodies and cytokines present in milk during *S. aureus* acute or

chronic infection are important tools to identify bacterial antigens relevant to disease. Immunoproteomics utilizes a combination of proteomics and immunoblotting to identify antigenic proteins and factors. SERPA combined with immunoproteomics has been employed to identify antigenic proteins using serum from infected cows with subclinical mastitis [246,247]. In addition, various bioinformatics tools, such as reverse vaccinology and *in-silico* approaches, have also been explored to analyze the surface proteome of *S. aureus* with a focus on vaccine antigenic targets [26,248,249]. Important contributions have been made using these techniques, however more immunologically extensive and disease-, as well as host-, specific approaches are needed for an effective *S. aureus* vaccine against bovine mastitis.

In this study, our aim was to use mastitic milk antibodies to identify relevant antigens for the prevention of bovine mastitis. Here we report that 2DE, coupled with immunoblotting and mass spectrometry, promoted the identification of immunogenic vaccine candidates from *S. aureus*. We characterized and prioritized these candidates *in silico,* and cloned and purified the antigens, EsxA and IsdC. EsxA and IsdC were confirmed by ELISA to be immunogenic and surface exposed during *S. aureus* infection of the udder. In addition, sequence analysis and PCR on genomic *S. aureus* DNA indicated that EsxA and IsdC were conserved and the presence of these antigens is found on the majority of bovine isolates. This is the first known report of the use of immunoproteomics to identify *S. aureus* antigens using milk from cows with mastitis.

Materials and Methods

Bacterial Strains, Culture Conditions and Milk Samples

The *S. aureus* strains used in this study were Newbould 305 [208], a clinical strain (C1, kindly donated by M. McGuire) and 37 additional clinical bovine *S. aureus* isolates (kindly supplied by Udder Health Systems Inc, Meridian, ID). Bovine *S. aureus* was characterized and confirmed by growth and haemolysis on MP2 agar (Udder Health Systems, Inc.), coagulase production, and 16s rRNA sequencing. For genomic DNA isolation, *S. aureus* was grown in Luria Broth (LB) with shaking at 37°C. For cell wall protein and surface adhesin expression, *S. aureus* was grown in low iron media (LIM) [250] with shaking at 37°C. For use in 2DE, milk from four different clinically mastitic cows was obtained from a diagnostic laboratory after confirmation of the presence of *S. aureus* by mass spectrometry (MALDI, Udder Health Systems Inc., Meridian, ID). Milk was pooled and 10 and 100µl was plated on MP2 agar for CFU determination ($1.7x10^7$ CFU/ml), *S. aureus* isolation, and confirmation as described above. Commercially available pasteurized milk, and pooled unpasteurized milk obtained from 5 *S. aureus* negative cows, was used as negative milk for comparison in 2DE. Unpasteurized milk obtained from five *S. aureus* infected cows and five *S. aureus* uninfected cows was used for ELISA. Collection and processing of milk for ELISA and negative control milk is described previously [184].

Isolation of *S. aureus* Cell Wall Associated Proteins

Cell wall associated proteins were isolated using trypsinization as reported [21]. Briefly, *S. aureus* Newbould 305, *S. aureus* C1, and *E.coli* (DH10-β Top10, Thermo Fisher) were grown in LIM overnight to an O.D. of 0.75-1.2 and harvested by

centrifugation (6000 ×g for 10 min at 4°C), prior to washing three times with 1x PBS (Table 1). Cells were then resuspended in 1/50 volume of the original culture in1x PBS containing 40% sucrose (pH 7.4) for 5 mins. Digestion was carried out with the addition of 50 µg of trypsin plus 2 mM DTT for 1hr at 37°C. The digested mixture was centrifuged at 3500 ×g for 10 min, and the supernatant with peptides was filtered with a 0.22 µm filter. Protease reactions were stopped with protease inhibitor solution (Halt Protease Inhibitor Cocktail 100X, Thermo Fisher Scientific, Waltham, MA). Total protein was analyzed by SDS-PAGE and measured for concentration using BCA (Pierce BCA protein assay kit, Thermo Fisher).

Table 4-1. Bacterial strains, plasmids and primers used in this study.

Bacterial strains			
	Genotype/relevant information		**Reference or source**
S. aureus Newbould 305	Bovine clinical isolate		[208]
S. aureus Clinical	Bovine clinical isolate		This study
E.coli Top10	DH10β		Invitrogen (Thermo Fisher)
Plasmids			
	Gene	**Vector**	**Reference or source**
pNM002	*esxA* (Newbould 305)	pTRCHisA	This study
pNM003	*isdC* (Newbould 305)	pTRCHisA	This study
Primers			
PCR		**Amplicon (bp)**	**Source**
*esx*A: **FW**GGCAATGATTAAGATGAGTCC **RV**GCAAACCGAAATTATTAG		287	This study
*isd*C **FW**GAGTATCGAAGGACATAAAG **RV**GCTAAGGATGCAACTGG		384	This study

Clones	**Gene**	**Amplicon (bp)**	**Source**
pNM002: **FW**GCTTCCGGGATCCGCAATGATTAAGATGAGTCCAG **RV**CAGTGCGGAATTCTTATTGCAAACCGAAATTATTAG	*esxA*	297	This study
pNM003: **FW**GCTTCCGGGATCCGCAGATAGCGGTACTTTGAATTATG **RV**CAGTGCGAAGCTTTTATTCCACATTGCCTTTAG	*isdC*	606	This study
pCK001: **FW**GCTACTGGATCCGCGGCAACAGAAGCTACGAAC **RV**GTGCATAAGCTTTCAAGTTTTTGGTAATTCTTTAGC	*isdA*	852	[65]

Two-Dimensional Gel Electrophoresis (2DE) and Western Blotting

Trypsinized protein extracts were cleaned using the ReadyPrep 2-D Cleanup kit (Bio-Rad, Hercules, CA) following manufacturer's instructions, and resuspended in ReadyPrep rehydration buffer. A total of 150 µl of 165 µg/ml of protein was used to rehydrate a 7 cm immobilized pH gradient (IPG) strip (Bio-Rad, Hercules, CA), pH 4-7 for 24 hrs. Rehydrated IPG strips were then iso-electro focused (IEF) on the first dimension using the Protean IEF system (Bio-Rad) at cycles 250 voltage for 20 min linear followed by 4000 volts for 120 min and 4000 volts at 10,000V-hr rapid. After IEF strips were equilibrated using equilibration solution I and II (2-D Starter kit, Bio-Rad) for 15 min each. For second dimension separation, the equilibrated strips were run on 12% SDS-PAGE and stained with Coomassie blue. 2D gels were electro-transferred to a polyvinylidene difluoride (PVD) membrane and blocked using blocking buffer (0.05% Tween 20 + 5% skim milk + 1× PBS). Membranes were washed and blotted with pooled mastitic milk or *S. aureus* negative milk (1:5000, dilution). The membrane was developed using the ECL western blotting substrate (Pierce, Thermo Fisher) and exposed to film for 24 hours to detect chemiluminescence.

Reported data represent 5 independent blots performed using pooled mastitic milk on separated proteins from *S. aureus* Newbould 305. Additional blots for comparison were performed each at least twice, using: *1)* uninfected pasteurized milk on proteins from Newbould 305, *2)* pooled unpasteurized milk from 5 *S. aureus* negative cows on proteins from Newbould 305, *3)* pooled mastitic milk on trypsinized proteins from *E.coli*, and *4)* pooled mastitic milk on trypsinized proteins from *S. aureus* C1. Spots identified on the western blot film were compared and aligned to the Coomassie blue stained 2D gel

using ImageJ software (https://imagej.nih.gov/ij/) to enable the selection of proteins with the brightest spot. Only those proteins showing prominent bright spots on *S. aureus* Newbould 305 or C1 immunoblots using *S. aureus* infected milk were picked for identification by mass spectrometry.

Mass Spectrometry Analysis

The proteins aligned with the brightest spots on the western were picked using a spot picker and digested with trypsin as described with modifications [245]. Briefly, gel spots were destained in buffer (50 mM ammonium bicarbonate/50% acetonitrile, reduced in 10 mM DTT) for 60 min at 56^{o}C, and alkylated in 55 mM iodoacetamide for 60 min at room temperature (RT) in the dark. Proteins were then digested with 20 µg/ml trypsin overnight at 30^{o}C. Peptides were extracted from the gel, dried under vacuum, and reconstituted in buffer (5% acetonitrile, 0.1% formic acid in water) for LC-ESI-MS/MS analysis. 5 µL of peptide mixture was injected onto a C18 reverse-phase column (10 cm x 75 µm, 3 µm, 120 Å). A linear gradient with two mobile phases at a flow rate of 300 nL/min was used to separate peptide mixtures. Full scan MS spectra were acquired from m/z 300-2000.

Collision-induced dissociation (CID) was used to fragment the precursor ions. MS/MS spectra were acquired in the data-dependent acquisition mode for the top ten most abundant precursor ions in the preceding full MS scan. Peptide spectral matching and protein identification were achieved by database search using Sequest HT and Mascot algorithms in Proteome Discoverer 1.4 (Thermo Scientific, Waltham, MA, USA). Raw spectrum data were searched against the UniProtKB/Swiss-Prot protein database for *S.aureus* (www.uniprot.org/ , obtained on June 14, 2016). Immunogenic proteins were

ranked on the basis of location and function using the PSORTb v3.0.2 (www.psort.org/psortb/) and UniProt (www.uniprot.org), and adhesion potential using Vaxign (www.violinet.org/vaxign/) (Table 2 and Supplementary Table 1).

Pcr, Cloning and Purification of Recombinant Proteins

Gene presence/absence was determined by electrophoresis of PCR products performed three times under optimized conditions. Primer an amplicon sizes are as indicated (Table 1). The plasmids; pNM002 and pNM003 were constructed for the expression of His-EsxA and His-IsdC. Corresponding primers for *isd*C and *esx*A (Table 1, restriction sites underlined) were used to PCR amplify genomic DNA from *S. aureus* Newbould 305. The resulting product was cloned into pTrisHisA (Invitrogen, Carlsbad, CA) and plasmids were transformed into *E.coli* Top10 (Thermo Fisher). Plasmids were confirmed by sequencing through the junctions. Transformed cells were induced and proteins were isolated from the bacterial cytosol for purification by cobalt affinity chromatography (Talon Metal Affinity Resin; Clontech Laboratories, Mountain View, CA) under denaturing conditions. Purified proteins were dialyzed against phosphate-buffered saline (1x PBS) with 5% glycerol, analyzed by SDS-PAGE, and confirmed by mass spectrometry. Protein concentrations were determined by BCA as described above.

ELISA

ELISA was used to detect antigen-specific antibody responses in milk from five *S. aureus* infected cows and compared to five uninfected controls. Microtiter plates (Nunc, Rochester, NY) were coated with 10 μg of purified protein (EsxA and IsdC, described above) per well in 1x PBS, blocked with buffer (1% skim milk + 1x PBS), and incubated with 2-fold dilutions of bovine milk, starting at 1:10 dilution, for 12 hr at 4°C.

Plates were then washed with PBS-T (1× PBS + 0.05% Tween 20), incubated with HRP-conjugated anti-bovine IgG (1:10,000; Thermo Scientific, Rockford, IL) in blocking buffer for 1 hr at 37°C, washed again and developed with tetramethylbenzidine (TMB One, Promega, Madison, WI). IgG titers were calculated after background values (protocol minus samples) were subtracted. Endpoint titers were defined as the reciprocal of the dilution giving an OD of 0.2, and results are representative of the assay performed independently three times.

Data and Statistical Analysis

Graphing and statistical analysis was performed with *JMP* SAS software (Cary, NC). Significance for ELISA was assessed using two-tailed Student's t-test for unequal variance. Values are reported as $p \leq 0.05$(*). Decoy database search was performed to calculate false discovery rate (FDR) of mass spectrometry data. Proteins containing one or more peptides with FDR≤0.05 were considered positively identified and reported.

Results

Extraction of Cell Wall Associated Proteins

The *S. aureus* surface proteome was used as the target for immunoproteomics in this study. Figure 4-1 illustrates the method of preparation of protein samples used to promote reliable identification of immunogenic surface or cell-wall bound proteins. *S. aureus* was cultured in low iron media (LIM) for the induction of virulence-related surface/cell wall anchored proteins and collected during late exponential and early stationary phase. Cells were trypsinized to release the cleaved surface fraction and separated in the first dimension by iso-electro focusing (IEF). After second-dimension separation, immunoproteomic analysis was conducted by performing a western blot on

the 2DE gel using *S. aureus* infected pooled milk. The goal of these methods was to detect surface exposed *S. aureus* proteins that induced humoral responses in the mastitic milk of infected cows.

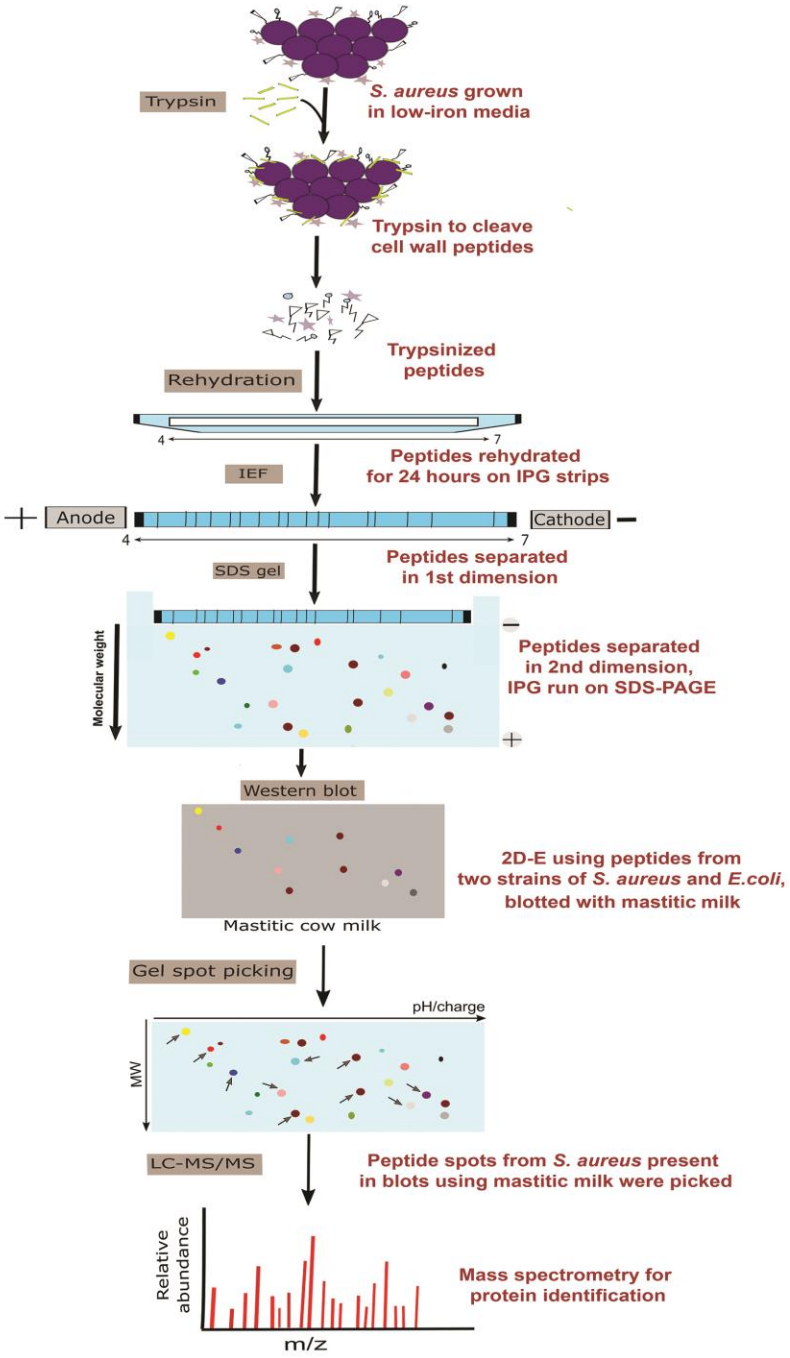

Figure 4-1. Diagram of the immunoproteomics techniques used for detection of immunogenic surface proteins from *S. aureus*. The experimental workflow, including: 2DE, immunoblots and mass spectrometry analysis, for identifying bovine disease relevant antigens is shown.

Identification of Immunogenic Antigens

S. aureus Newbould 305, an *S. aureus* bovine clinical isolate, and *E. coli* (DH10-β) surface proteins were trypsinized and extracted and first run in one dimension on SDS-PAGE (Figure 4-2A, lanes 1 and 2). The isolated proteins were then rehydrated and separated on a two-dimensional gel, as shown in Figure 4-2B and D. In order to assess the immunogenic proteins from *S. aureus* as previously mentioned, trypsinized proteins were subjected to western blot. To limit the selection of cross-reactive proteins, *E.coli* surface proteins were also extracted and subjected to western blot analysis using mastitic cow milk as a negative control. As expected, mastitic milk recognized more proteins from the *S. aureus* extract (Figure 4-2C). Several highly immunogenic areas (containing multiple spots) were detected only in *S. aureus* blots and not in the *E. coli* blot (Figure 4-2C and E). Very few immunogenic proteins were detected in the control milk western blots. The anti-staphylococcal antibody repertoires from mastitic cows were deemed to be unique and a relevant tool for further analysis.

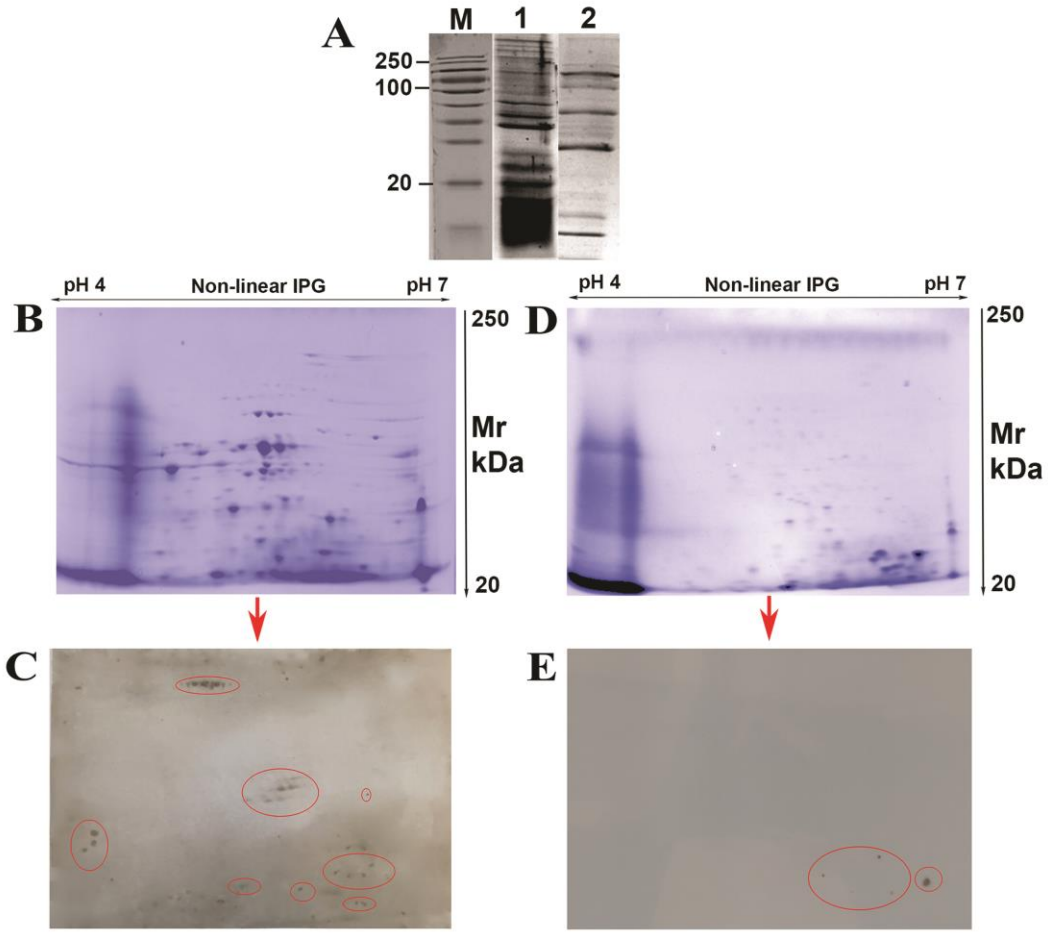

Figure 4-2. Representative SDS-PAGE, 2DE and western blots showing the workflow to the selection of immunogenic spots (A) The 1-dimensional SDS-PAGE image of *S. aureus* Newbould 305 trypsinized protein (lane 1) and *E. coli* trypsinized protein (lane 2). (B) 2DE gel conducted on 7 cm, pH 4-7, IPG strips on *S. aureus* trypsinized protein. (C) Immunoblot of *S. aureus* 2DE using pooled bovine mastitic milk, with the immunogenic region highlighted in red circles. (D) 2DE gel conducted on 7 cm, pH 4-7, IPG strips on *E. coli* trypsinized protein. (E) Immunoblot of *E. coli* 2DE using bovine mastitic milk, with the immunogenic areas of the blot highlighted in red circles.

LC-MS/MS and *in silico* Analysis

2DE was performed five times, using trypsinized proteins from Newbould 305, a clinical bovine mastitis *S. aureus* isolate and *E. coli*. The collected immunogenic spots corresponding to western blots were picked and identified by tandem mass spectrometry. An uninterpreted database search approach using Sequest and Mascot algorithms was employed to identify peptides and proteins. The complete set of identified proteins using this approach is listed in Table 4-4 and ranked by coverage, location and function. The most promising immunogenic proteins for incorporation into a bovine vaccine were further ranked on the basis of location and function using the PSORTb, UniProt and Vaxign online tools (Table 4-2) [251–253]. Two specific surface exposed proteins and virulence factors, IsdC and EsxA, were targeted for further characterization. Figure 4-3 shows a representative peptide spectral match of the isolated IsdC and EsxA 2DE peptides.

Table 4-2. Selected antigens on the basis of coverage, function, adhesion probability and location.

Gene	Protein name	Coverage	Function	Location	Adhesion probability
isdC	Iron-regulated surface determinant protein C	29.07%	Hemin binding, and haem transfer	Cell wall	57%
esxA	ESAT-6 secretion system extracellular protein A	89.6%	Virulence factor	Extracellular	43%
isaA	Immunodominant staphylococcal antigen A	15.02%	Hydrolase activity, acts on glycosyl bonds	Extracellular	71.9%
lukL1	Uncharacterized leucocidin like protein 1	2.66%	Pathogenesis	Extracellular	59.4%
fmtA	Methicillin-resistance factor	4.28%	Antibiotic resistance	Cell membrane	39.8%
spA	Immunoglobulin G-binding protein A	18.8%	IgG binding, immune evasion	Cell wall	53%
feoB	Ferrous iron transport protein B	3.01%	Ferrous iron transmembrane transporter activity	Cell membrane	34.1%
map	MHC Class II analog protein	1.89%	Modulating host immunity	Cell membrane	51.7%

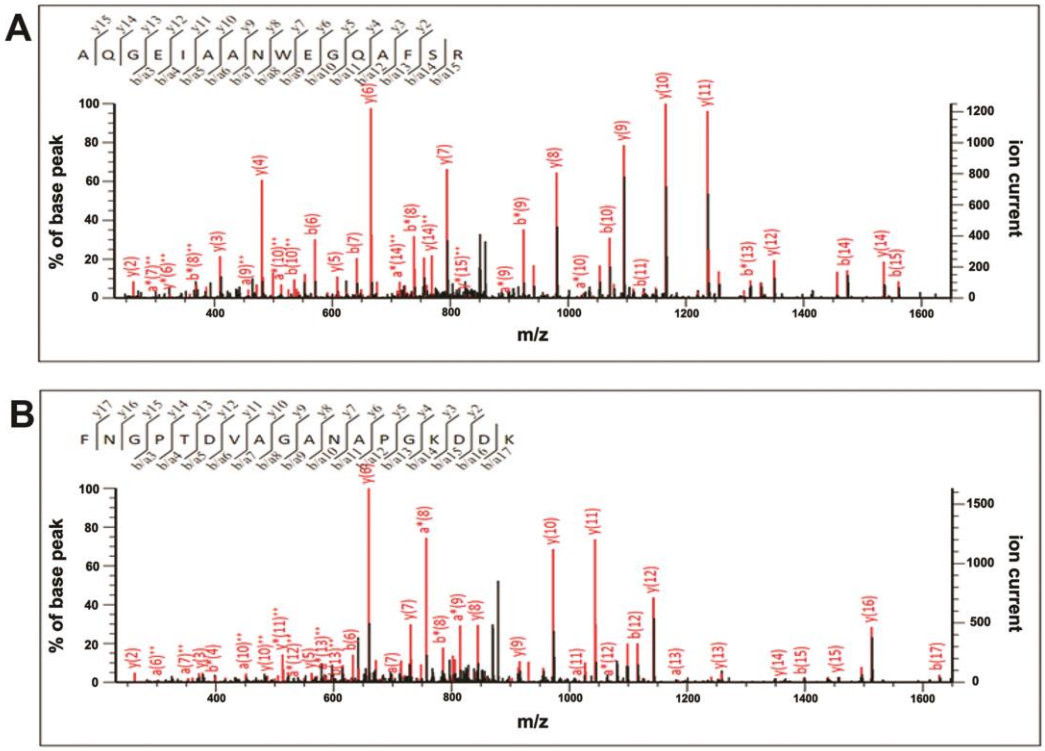

Figure 4-3. Representative MS/MS spectra of peptides identified by Mascot. (A) MS/MS fragmentation of AQGEIAANWEGQAFSR found in the ESAT-6 secretion system extracellular protein (EsxA); (B) MS/MS fragmentation of FNGPTDVAGANAPGKDDK found in the Iron-regulated surface determinant protein C (IsdC).

Conservation and Prevalence of Antigens

To determine if IsdC and EsxA are conserved at the amino acid level, phylogenetic analysis was completed using 50 published sequences (www.ncbi.nlm.nih.gov) as shown in Figure 4-4. Results include five known cow isolates and percent identity is compared to *S. aureus* Newbould 305. Sequences of both proteins are highly conserved, with EsxA 100 % conserved among *S. aureus*. PCR was performed to estimate the genetic prevalence of *isd*C and *esx*A in bovine isolates of *S. aureus*. Gene-specific primers (Table 4-1) were used to amplify the genes from 37 clinical bovine *S. aureus* isolates and compared previously reported analysis of *isd*A [184]. As shown in

Table 4-3, The results indicate that the presence of *isd*C and *esx*A is 92% conserved in bovine *S. aureus* isolates.

Gene	*isd*C	*esx*A	*isd*A[a]
# positive isolates	34	34	36
# strains tested	37	37	37
Percent conservation	92%	92%	97%

[a] as reported previously [184]

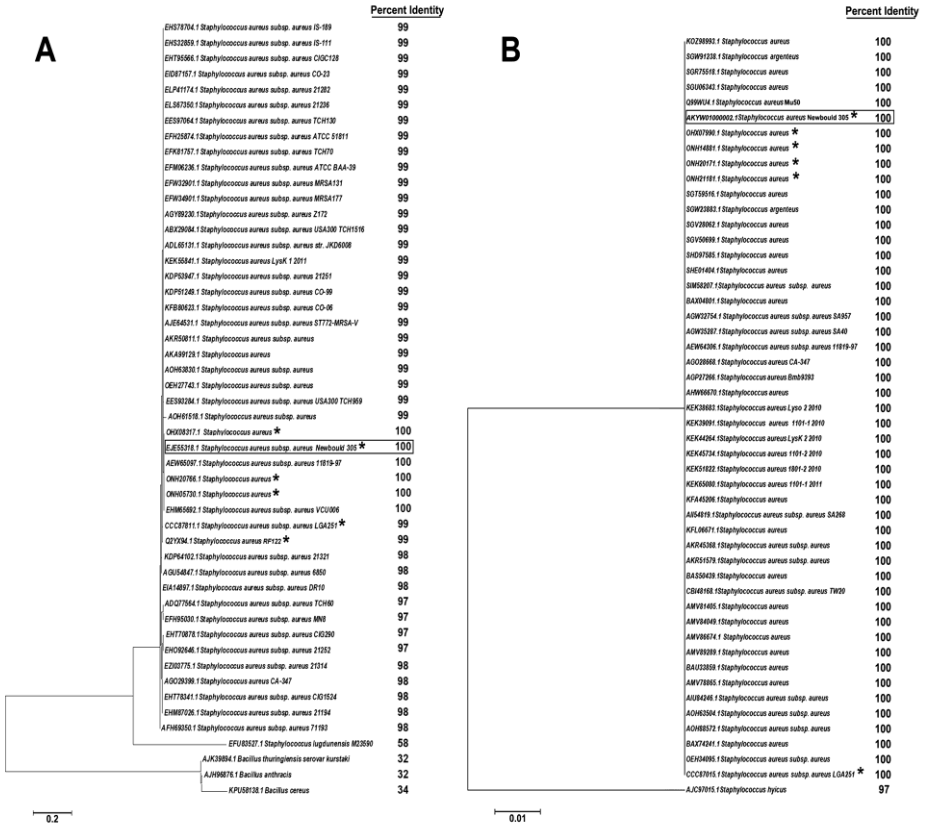

Figure 4-4. Dendrogram of pairwise amino acid alignments of (A) IsdC and (B) EsxA from 50 published sequences. Evolutionary analysis was performed with the Maximum Likelihood method based on the JTT matrix-based model (MEGA, Version 7). Percent identity to the sequence from the bovine Newbould 305 *S. aureus* strain is shown. Isolates from cow (*) are indicated

Cloning, Expression and Immunogenicity of Antigens

*isd*C and *esx*A were cloned into a 6x histidine vector to construct the expression plasmids, pNM003 and pNM002 (Figure 4-5A and C). IsdC (~25.5 kD) and EsxA (~14.5 kD) proteins were then expressed and analyzed on SDS-PAGE (Figure 4-5B and D). Purified proteins were then used to confirm the immunogenicity of IsdC (Figure 4-6B) and EsxA (Figure 4-6C) by ELISA using *S. aureus* culture positive or negative milk from 5 different cows. Immune responses to the previously characterized IsdA were also determined for comparison (Figure 4-6A) [184]. IsdC and EsxA were found to stimulate significant IgG antibody titers in mastitic milk as compared to uninfected milk. Results indicate that IsdC and EsxA are immunogenic and expressed *in vivo* in the bovine udder.

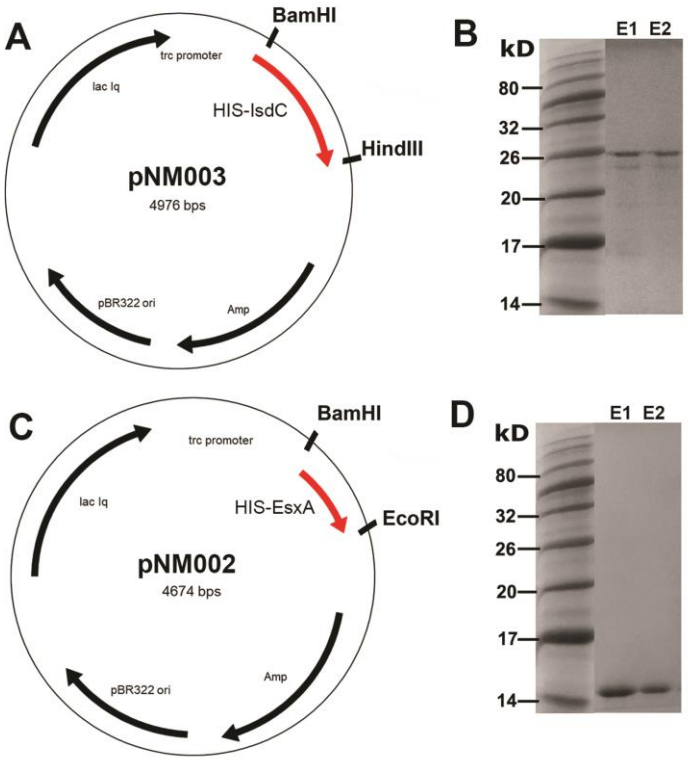

Figure 4-5. Expression and purification of IsdC and EsxA proteins. (A) **Structure and operon organization of pNM003 for His-IsdC expression and** (B) **SDS-PAGE of purified IsdC (~25.5 kDa).** (C) **Structure and operon organization of pNM002 for His-EsxA expression and** (D) **SDS-PAGE of purified EsxA (~14.5 kDa). E1= elution 1, E2 = elution 2.**

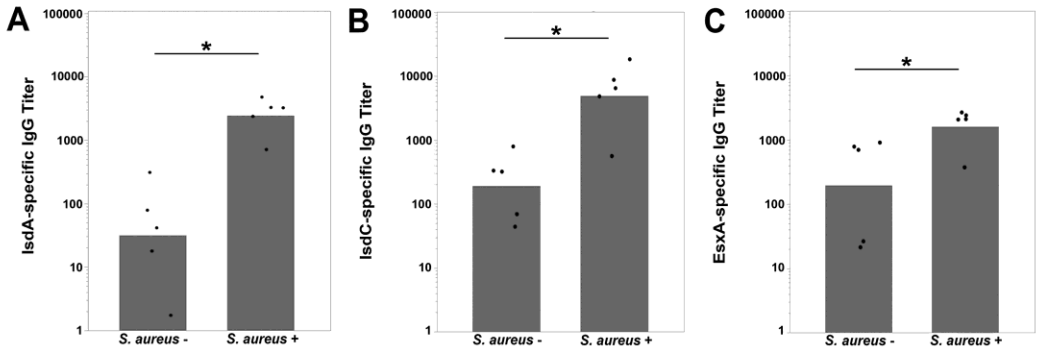

Figure 4-6. Antigen-specific IgG responses from S. aureus culture positive and S. aureus culture negative bovine milk. (A) anti-IsdA, (B) anti-IsdC, and (C) anti-EsxA IgG responses. Data are presented as the log transformed geometric mean of independent titers from five cows. Significance (*) of anti-IsdA (p=0.014), anti-IsdC (p=0.035) and EsxA (p=0.019) using a two-tailed Student's t-test for unequal variance.

Discussion

Bacterial surface proteins are critical immunodominant antigens that are often the first molecules to interact with host cells and tissues. These proteins are especially important targets for *S. aureus* vaccine development as they are also key for adhesion and nutrient acquisition, and their presence and structure more conserved across strains than the polysaccharide capsule [254]. In this study, our approach was to identify immunogenic cell wall associated and surface proteins for characterization as vaccine antigens to prevent *S. aureus* bovine mastitis. The presence of anti-staphylococcal IgG antibodies in milk during subclinical and clinical bovine mastitis has been reported [92]. Here, we report a unique immunoproteomics strategy to detect antigenic staphylococcal proteins using IgG present in milk. A total of 38 unique and immunogenic *S. aureus* proteins were identified, out of which 17 proteins were cell wall associated, or extracellular, and previously reported as involved in virulence. Eight proteins were further ranked based upon their subcellular location, coverage and function and two of them, IsdC and EsxA, purified for confirmation of immune responses [21].

Despite taking precautions to minimize cell lysis, this approach did also identify intracellular or cytosolic proteins including: elongation factors and ribosomal proteins (Supplementary Table 1). Adjustment of trypsinization time and conditions did not eliminate the presence of these proteins, and the detection of cytosolic proteins is a recognized limitation of immunoproteomics [255]. While immunogenic cytosolic proteins can be potential vaccine antigens, they may also be detected due to abundance, and thus we did not pursue the characterization of them [256]. Phase of growth is also a key consideration for immunoproteomics. We targeted the late exponential phase and early stationary phases of growth (0.75-1.2 OD_{600} in LIM) as previous reports, and our studies, have indicated this range is optimal for *isd*A and other virulence factor expression [257]. IsdA is a well characterized immunogenic surface adhesin from *S. aureus*, and we, unexpectedly, did not identify this protein using these techniques [258]. Lack of identification of IsdA could be due to trypsinization methods that result in aberrant IsdA cleavage, lack of release of IsdA due to surface hydrophobicity, or low peptide concentration that was not detected or not fully resolved from other spots/proteins [259]. We attempted to isolate surface proteins using alternate techniques, including: lysostaphin and silica beads (*data not shown*), but these efforts resulted in the identification of many more cytosolic proteins. Trypsinization enables extraction of surface proteins effectively but is limited to exposed lysine and arginine amino acid residues on the surface. Despite these limitations, a number of immunogenic *S. aureus* virulence factors that may represent bovine vaccine candidates were identified, and results emphasize the significance of milk antibodies for the identification of antigens exposed during udder colonization and mastitis.

Out of the top eight identified proteins, IsdC and EsxA were selected for further analysis on the basis of coverage by mass spectrometry, cell wall location and function in relation to virulence and pathogenesis. IsdC and EsxA were tested for reactivity to bovine milk from *S. aureus* infected cows as an indicator of surface exposure *in vivo*. In comparison to the IsdA antigen, which has been reported to induce antibody production during *S. aureus* mastitis, EsxA and IsdC were also immunogenic [184]. These proteins have been studied and described as virulence factors for *S. aureus* pathogenesis. EsxA is a surface exposed *S. aureus* protein as determined by trypsinization and mass spectrometry [260]. This protein is also an important factor for pathogenesis and abscess formation in mice [261]. A recent report identified anti-EsxA antibodies in patients infected with antibiotic resistant strains of *S. aureus* [81]. EsxA has also been evaluated as a vaccine candidate, and induces Th1 and Th17 immune responses against invasive *S. aureus* in murine models [82,262]. IsdC is a surface adhesin from the Isd (iron regulated surface determinant) family that is required for iron sequestration and ECM binding. IsdC is involved in haem transfer from the membrane to the cytosol and important, but not essential, for *S. aureus* iron uptake [263]. IsdC contains one near-iron-transporter (NEAT) domain similar to IsdA, and may contribute to biofilm formation through dimerization [84]. In this study, we found both IsdC and EsxA to be highly conserved at the amino acid level using published genomes, and *isd*C and *esx*A were present in 92% of tested bovine *S. aureus* isolates.

Other proteins identified using this technique also represent interesting vaccine candidates, including: FeoB, Map, SpA and IsaA [248,264–268]. These proteins were not currently selected for further analysis due to significantly lower coverage by mass

spectrometry. However, a number of them have been pursued as human vaccine candidates and may also represent bovine candidates. While additional studies are required to assess the specific immunogenicity and protective efficacy of IsdC and EsxA in bovines, the methods described here are unique and represent a useful screen to identify bovine mastitis vaccine candidates.

Table 4-4. *S. aureus* proteins identified as ranked by mass spectrometry coverage

Accession No.	Protein name	Symbol	Coverage	# AAs	Function	Location	Trans-membrane helices
Q99WU4	ESAT-6 secretion system extracellular protein A	ESXA	89.69	97	Virulence factor	Extracellular	0
Q2FJN4	Alkyl hydroperoxide reductase subunit C	AHPC	40.21	189	Peroxidase activity	Cytoplasm	0
A7X3V3	UPF0342 protein SAHV_1830	Y1830	32.46	114	unknown	Cytoplasm	0
A6U0U8	Iron-regulated surface determinant protein C	ISDC	29.07	227	Hemoglobin binding/metal ion binding	Cell wall, extra cellular	2
Q2FEQ7	50S ribosomal protein L30	RL30	23.73	59	Structural constituent of ribosome	Cytoplasm	0
Q5HG56	Probable tautomerase SACOL1399	Y1399	19.67	61	Isomerase activity	Cytoplasm	0
P02976	Immunoglobulin G-binding protein A	SPA	18.80	516	IgG binding	Cell wall	1
P60157	Probable transglucosylase IsaA	ISAA	15.02	233	Hydrolase activity, acting on glycosyl bonds	Extracellular	0
P99075	Fructose-bisphosphate aldolase	ALF2	9.79	286	Fructose-bisphosphate aldolase activity	Cytoplasm	0
A6U135	Methionyl-tRNA formyl transferase	FMT	8.68	311	Methionyl-tRNA formyl transferase activity	Cytoplasm	0

A6U174	30S ribosomal protein S2	RS2	8.24	255	Structural constituent of ribosome	Cytoplasm	0
P0AEZ5	Septum site-determining protein MinD	MIND	7.04	270	ATPase activity	Cell membrane	0
P39851	Putative tyrosine-protein kinase CapB	CAPB	4.82	228	ATP binding	Cytoplasmic Membrane	0
P66816	NAD-dependent protein deacetylase	NPD	4.53	243	Metal ion binding	Cytoplasm	0
Q6GI27	Protein FmtA	FMTA	4.28	397	Antibiotic resistance	Cell membrane	1
P63582	Arginosuccinate lyase	ARLY	4.14	459	Arginosuccinate lyase activity	Cytoplasm	0
P35272	Chain length determinant protein	WZZB3	4.00	325	unknown	Cell inner membrane;	2
Q8FB30	tRNA-dihydrouridine	DUSA	3.93	331	Flavin adenine dinucleotide binding	Cytoplasm	0
P37386	Probable cadmium-transporting ATPase	CADA2	3.86	804	ATP binding	Cell membrane	7
Q2YU20	Putative multidrug export ATP-binding/permease protein SAB1799c	Y1799	3.81	578	ATPase activity, coupled to transmembrane movement of substances	Cell membrane	5
Q2YUG0	tRNA N6-adenosine threonyl carbamoyl transferase	TSAD	3.52	341	metal ion binding	Cytoplasmic	0
Q6G845	UPF0421 protein SAS1811	Y1811	3.35	328	unknown	Cell membrane	4
Q2FVZ4	Lipid II: glycine glycyltransferase	FEMX	3.33	421	Transferase activity, transferring amino-acyl groups	Cytoplasm	0
P58995	dITP/XTP pyrophosphatase	IXTPA	3.08	195	metal ion binding	Cytoplasm	0
Q5HD01	Fe (Ferrous iron transport protein B)	FEOB	3.01	664	Ferrous iron transmembrane transporter activity	Cell membrane	10
Q5HE97	ATP synthase subunit beta	ATPB	2.98	470	ATP binding	Cell membrane	0

Q2FWE3	Low molecular weight protein-tyrosine-phosphatase PtpB	PTPB	2.88	139	Protein tyrosine phosphatase activity	Cytoplasm	0
P21224	Uncharacterized leukocidin-like protein 1	LUKL1	2.66	338	Pathogenesis	Extracellular	0
Q99V41	Bifunctional autolysin [Includes: N-acetylmuramyl-L-alanine amidase	ATL	2	1248	Amidase activity	Cell wall	0
Q5HGM7	Orotate phosphoribosyl transferase	PYRE	1.97	203	Orotate phosphoribosyl transferase activity	Cytoplasm	0
P72367	Capsular polysaccharide type 8 biosynthesis protein cap8A	CAP8A	1.80	222	Sugar efflux transmembrane transporter activity	Cell membrane	2
Q6GDD3	Lipase 1	LIP1	1.62	680	Metal ion binding	Extracellular/ membrane	1
A6U074	ATP-dependent helicase/nuclease subunit A	ADDA	1.56	1217	ATP binding	Cytoplasm	0
Q6GBT3	Probable branched-chain-amino-acid aminotransferase	ILVE	1.40	358	L-isoleucine transaminase activity	Cytoplasm	0
Q69HT9	Multicopper oxidase	MCO	1.34	447	Copper ion binding	Cytoplasm	0
Q5HG29	Conserved virulence factor B	CVFB	1.33	300	unknown	Cell membrane	0
Q6GGU4	3-dehydroquinate synthase	AROB	1.13	354	3-dehydroquinate synthase activity	Cytoplasm	0
Q7A695	N5-carboxyaminoimidazole ribonucleotide synthase	PURK	1.07	374	5(carboxyamino)imidazole ribonucleotide synthase activity	Cytoplasmic Membrane	0

CONCLUSIONS AND INTERPRETATIONS

The chapters of this work focus on evaluating various aspects of designing a mucosal vaccine against *S. aureus* mastitis. We hypothesized that a *mucosal, enterotoxin-based vaccine containing multiple relevant antigens will protect cows against S. aureus mastitis*. To being to address this hypothesis and construct a vaccine for bovine mastitis, experimentation focused on three main areas: *1)* characterizing the IsdA antigen for its intra-strain conservation, ECM binding, and immune reactivity for use as a bovine vaccine candidate, *2)* analyzing the immunogenicity and safety of a cholera toxin based subunit vaccine (IsdA-CTA_2/B + ClfA-CTA_2/B), and *3)* developing an immunoproteomics approach to discover new proteins from *S. aureus* that can be incorporated into the vaccine. As discussed in Chapter 1, deteriorating animal health and economic losses caused by bovine mastitis is a worldwide concern. *S. aureus* caused mastitis is of critical importance due to refractory antibiotic treatments and the growing incidence of antibiotic resistance. Additionally, the contagious nature of *S. aureus* caused mastitis and ability to establish subclinical infection make prevention of this disease a top priority. An effective vaccine against this pathogen that can prevent mastitis caused by different strains and serotypes will reduce the use of antibiotics, improve animal health, reduce economic losses and also provide valuable information for human *S. aureus* vaccine development.

IsdA has been previously described as an important vaccine antigen and is also used in potential human vaccines with other antigens. However, the characterization of

IsdA specifically as a bovine antigen has not been reported and is explored here for the first time. It is known that, due to intra-strain variability, the conservation of antigens is critically important for a vaccine against a pathogen like *S. aureus.* As discussed in Chapter 2, a main focus of this research was to identify the genetic prevalence and expression of IsdA from bovine *S. aureus*. Our results indicate that IsdA is present in 28 strains from a total of 29, making it 97% percent conserved (Table 2-2, Chapter 2). In addition to IsdA we also detected prevalence of a number of other surface adhesins from *S. aureus* (Table 2-2, Chapter 2). Immunogenicity assays from this study revealed that the IsdA antigen can stimulate antigen-specific active antibodies that were capable of opsonophagocytosis, supporting the conclusion that IsdA is a promising bovine vaccine antigen. Multiple alignment of the IsdA protein revealed the presence of two different variants of IsdA (represented by strains Newbould 305 and MRSA252). Four amino acid differences observed at the C-terminal of these two variants of IsdA mediated variable binding preferences to the host ECM molecules FN and FG (Figure 2-2, Chapter 2). The NEAT domain of IsdA is known to coordinate FG binding, however, the role of the C-terminal in ECM binding was identified in this study for the first time.

The studies described in Chapter 2 determined that IsdA is expressed in milk *in vivo* during active *S. aureus* infection. To detect the level of expression of *isd*A and other antigens *in vitro* we performed additional qRT-PCR analysis of *isd*A, *clf*A, *isd*B and *flb*A under the iron limiting conditions of milk using *S. aureus* (Newbould 305), *S. aureus* (MRSA USA300) and *S. haemolyticus.* These results revealed that there is increased expression of *isd*A, *isd*B and *clf*A in the iron limiting conditions of milk from *S. aureus* (305 Newbould) and *S. aureus* (MRSA), but not *S. haemolyticus*. Although the

expression was increased for all three adhesins, *isd*A and *isd*B showed higher expression than *clf*A (Appendix A, Figure A-1). Furthermore, we conducted growth curve analysis on all the three strains, to detect if the change in expression in milk is solely due to growth phase changes, and the results indicated partial interdependence of expression on growth phase as well as iron limitation (Figure A-2, Appendix A). In addition, we identified enhanced binding of bovine strains of *S. aureus* (305, Newbould) to FN in milk as compared to LB (Figure A-3, Appendix A).

The data presented in Chapter 2 and Appendix A highlight that IsdA is an important surface adhesin for *S. aureus* and a promising candidate for bovine vaccines. Although IsdA is likely to be highly conserved, future studies with a larger number of strains, from different geographical regions, will be important, for better characterization of this antigen. Also, additional *in vitro* gene expression studies in low iron conditions of milk with immunogenic proteins (IsdC, EsxA and SpA) will be valuable to understand their roles in bovine mastitis. The ECM binding assays of our study, revealed higher binding of bovine strains to FN in the presence of milk, however, binding with other ECM molecules like laminin and collagen IV should be evaluated, as these molecules are also abundant in bovine udder and are involved in mammary gland development. Lastly, creating a *isd*A targeted gene mutant to study the pathogenicity and ECM binding of this antigen *in vitro* and *in vivo* might be the gold standard, however, redundancy of *S. aureus* adhesins have in the past been shown to be a problem for analysis in mutational studies.

As discussed in Chapter 3, two field trials on the mucosal subunit vaccine containing IsdA and ClfA (IsdA-CTA_2/B and ClfA-CTA_2/B) were conducted. The trails differed in the amount of vaccine administered and the number of booster doses

thereafter. Results indicate that the stimulation of significant immune responses occurred in vaccinated animals and a slight but effective booster response occurred that produced antibody responses prior to freshening. Trial 2 in these studies had a higher dosage of vaccine from the start which contributed to higher immune responses throughout the 50-day vaccination trial period. IsdA and ClfA were able to induce antigen-specific antibodies, however, IsdA consistently showed higher and more significant immune responses. The vaccine induced significant humoral and cellular immune responses in serum, and while significance responses in milk were not detected by ELISA, data supportive. Increased expression of IL-4 cytokines was determined on day 60 of Trial 2, indicating that this vaccine induces aTh2 bias. Importantly, serum and milk antibodies stimulated by the vaccine were able to successfully induce opsonophagocytosis (Figure 3-3 and 3-4, Chapter 3). These results provide evidence that induced antibodies were active and specific to the antigen, which is key for a successful *S. aureus* vaccine. Antibodies stimulated in milk could also significantly induce opsonophagocytosis, despite being non- significant in ELISA immune assays, indicating that these important antibodies are also present and efficient in inducing bacterial killing (Figure 3-7, Chapter 3). Overall results from immunogenicity trials show that the vaccine is inducing effective immune responses, however the immune stimulation was lower in milk, indicating that there is a requirement for more exploration of additional antigens and the mechanisms involved. The intranasal route showed promising results, although other methods like intramammary or intravaginal routes of delivery might also be explored in the future. A challenge trial for this vaccine is underway, and although these studies are technically difficult, it might provide valuable information about the protective capabilities of the

vaccine. Further, a field trial with a larger number of cows and with newer immunogenic antigens will be important to detect the enhancement of the effectiveness of the multivalent vaccine. In addition, more knowledge on the critical role of specific antigen during mastitis can be evaluated on mastitic mouse models.

The mucosal vaccine analyzed in Chapter 3, was comprised of two antigens, and we aim to incorporate new antigens into this vaccine in the future. To identify new immunogenic proteins from the surface of *S. aureus*, we developed an immunoproteomics based approach as described in Chapter 4. This unique approach allowed us to detect immunogenic proteins from the surface of *S. aureus* by using milk antibodies (Figures 4-1, 4-2 and 4-3, Chapter 4). Two-dimensional electrophoresis coupled with western blotting and mass spectrometry, led to the identification of 54 proteins from *S. aureus* and 8 unique immunogenic surface proteins involved in virulence. Two of the eight proteins, IsdC and EsxA, showed high immunogenic potential and, with further characterization, can be incorporated into the vaccine (Figure 4-6, Chapter 4). Further analysis on 6 of the identified 8 surface proteins is underway through cloning and protein purification. After this characterization, these antigens can be potentially incorporated into the mucosal *S. aureus* vaccine. Although this technique enabled us to discover new antigens, few well-known immunogenic antigens like IsdB, ClfA, and FnbpA, were identified in these experiments, which suggests a limitation. This technique strongly relies on the capability of trypsin to shave off the surface proteins, which means cleaving only on lysine and arginine residues. Thus, important immunogenic proteins may have been missed. Future work for this aim may include the extraction of surface proteins from *S. aureus* under more stringent conditions of digestion

time, growth phase and concentration of trypsin to ensure less contamination of cytosolic proteins, and may also include the use of other enzymes like elastase and proteinase K for surface protein extraction by *S. aureus*.

The innovation and unique activity of this bovine *S. aureus* mucosal vaccine lies in its purified and multivalent nature, and its combination with a cholera toxin-based adjuvant. Cholera toxin adjuvant is a well-known and potent immunomodulator, that aids in antigen presentation, B cell isotype switching and translocation of co-administered antigens. This adjuvant, in combination with *S. aureus* surface proteins that are key targets for blockage, has the capacity to prevent bacterial colonization. While the protective efficacy of this vaccine is yet to be determined, the experiments described are essential steps in its progress and development, and these studies suggest it has the potential to improve animal health by preventing bovine mastitis caused by *S. aureus*. An effective vaccine would have positive impacts on milk production, antibiotic use, and agricultural economics and may also have lasting positive effects on human health and nutrition.

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APPENDIX A

The Low Iron Conditions of Milk Promote *Staphylococcus Aureus* IsdA Expression and Fibronectin Binding

Materials and Methods

Bacterial growth, RNA isolation and quantitative real-time PCR.

S. aureus Newbould 305, *S. aureus* USA 300 and *S. haemolyticus* (bovine, *isd*A+) were grown in Luria Broth (LB) to a bacterial count of 1x 10^8 cells. Cultures were harvested and grown microaerophilically in 5% skim milk (SKM), SKM + 200 μM $FeSO_4$, LB or Low Iron Media (LIM: 2 g NaCl, 1.2 g $NaHCO_3$, 1.6 g yeast extract, 6 g protease peptone to 400 mL) at 37°C (21). Optical density was determined at 600 nm every 4 hours. At 12 hr and 24 hr, cultures were centrifuged at 3000 Xg and the pellet was washed 2X in buffer (1X PBS + 0.5 mM EDTA). Total RNA was extracted from the cell pellet as described above for RT-PCR. RNA was suspended in 30 μl RNase free water and then stored at -80°C. RNA concentration and purity was confirmed by the 260/280 ratio (Nanodrop, Thermo-Fisher) and PCR reactions were conducted between each step to ensure the absence of genomic DNA. Total isolated RNA was used to synthesize first strand cDNA according to manufacturer's instructions (Revert Aid Kit, Thermo-Fisher). Resulting cDNA was diluted 1:500 with RNase free water prior to qRT-PCR. The qRT-PCR was conducted using SYBR FAST qPCR master mix (Kappa Biosystems, Wilmington, MA). All samples were assayed using standard cycling conditions of: denaturation at 95 °C for 3 min and 40 amplification cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec (Eppendorf Mastercycler Realplex^2). All reactions contained 2 μl of cDNA template, 10 μl of SYBR Green Master Mix (2X), 0.5 μl of 100 μM of each primer, and 7 μl of RNase free water. Fold changes were calculated using ΔΔCT method keeping 16S as normalizer. 16S RNA was the most stable housekeeping gene in our experiments. All the experiments were conducted with three

biological replicates repeated at least three times.

Fibronectin binding assays.

Black walled and clear walled (Nunc, Thermo-Fisher) ninety-six well plates were coated with 50 µl of 10 µg/mL fibronectin (R&D Systems, Minneapolis, MN) in 1x PBS, and incubated for 12 hr at 4°C, followed by two washes of 100 µl in 1X PBS. Plates were then incubated at 37°C with $1x10^8$ cells of *S. aureus* Newbould 305*, S. aureus* USA300*,* or *S. haemolyticus* (*isd*A+) in either LB versus SKM, or SKM versus SKM + 200 µM $FeSO_4$ for 12 hours. After incubation, the plates were washed with 100 µl of 1X PBS two times and 150 µl Alamar Blue dye (0.1% reazzurin in 1X PBS) was added to black walled plates for the fluorescent metabolic dye assay. Plates were read at 530/590 nm after 12, 18 and 24 hrs. Assays were also performed by adding 100 µl of top agar (10 g LB, 3 g agar to total 400 mL) to clear walled plates instead of Alamar Blue. Bacterial growth was quantified by the absorbance of top agar plates read at 600 nm after 12 and 24 hrs at 37°C. For serum-blocking, assays were performed as described above, however 1:100 dilution of heat inactivated immunized and mock immunized (1X PBS) mouse serum was mixed with $1x10^8$ cells of *S. aureus* MRSA252 that had been grown overnight in LIM. The mixture was incubated at 37°C for 30 mins and 100 µL was added to the ninety-six well plates containing 10 ug/mL fibronectin in 2-fold dilutions. Plates were washed and quantified with Alamar Blue as described. Pooled, day 14 anti-IsdA mouse serum was obtained after intranasal vaccination of 6 9-week old BALB/c mice that received purified IsdA (17 µg) plus CT (5 µg) adjuvant on days 0 and 10, as described previously (22).

Graphing and statistical analysis.

All graphing and statistical analysis was performed with *JMP* SAS software (Cary, NC) or R version 3863.0.2. PCRs, ELISAs and CFU assays were performed in triplicate. The ΔΔCT method was used to calculate fold changes for qPCR experiments. Significance was calculated using Tukey-Kramer pairwise comparison on *JMP* software using three biological and two test replicates. Significance between antibody responses, SCC and CFU counts were determined using the non-parametric Wilcoxon Rank Score (Mann-Whitney) test or between three groups using the Tukey-Kramer pairwise comparison. *P*-values are reported as $p < 0.05$(*) or $p < 0.01$(**) with a value of $p < 0.05$ considered statistically significant.

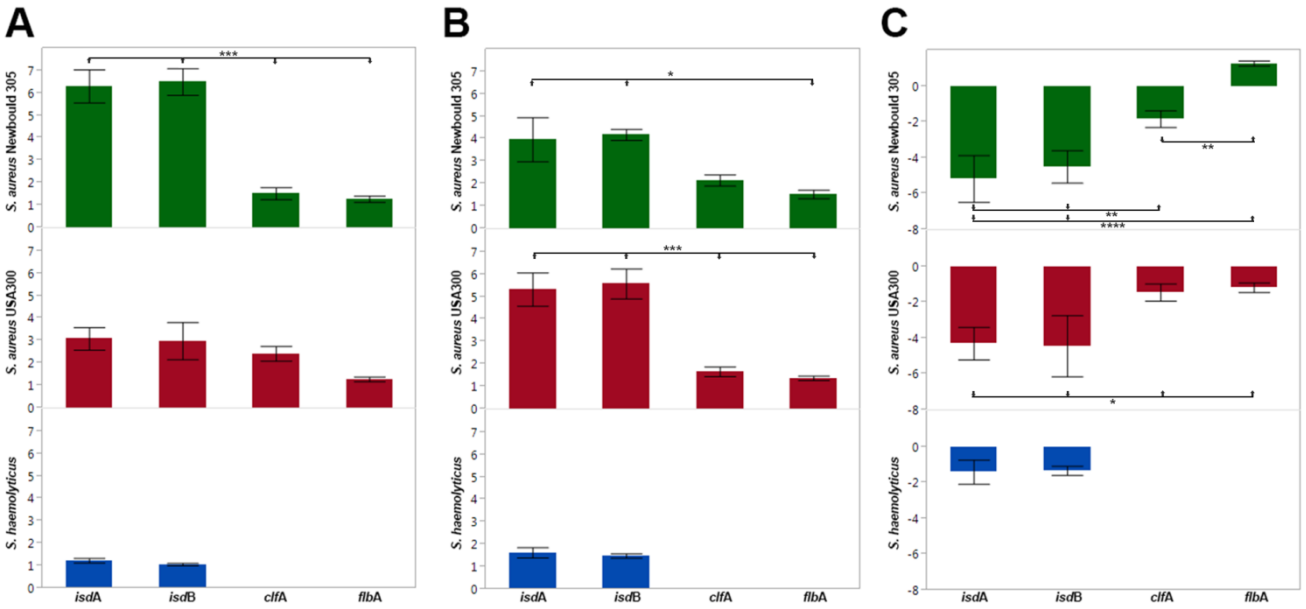

Figure A-1. Expression of staphylococcal adhesins *in vitro*. Quantitative RT-PCR showing normalized Ct values as fold change comparison of gene expression after growth of human and bovine *Staphylococcus* in A) 5% skim milk (SKM) compared to Luria broth (LB), B) low iron media (LIM) compared to LB and C) SKM plus $FeSO_4$ compared to SKM alone, after 24 hours at 37°C. Significance using the Tukey-Kramer pairwise comparison, $p \leq 0.05$(*), $p \leq 0.01$(**), $p \leq 0.001$ (***) or $p \leq 0.0001$ (****) between the expression of genes is shown.

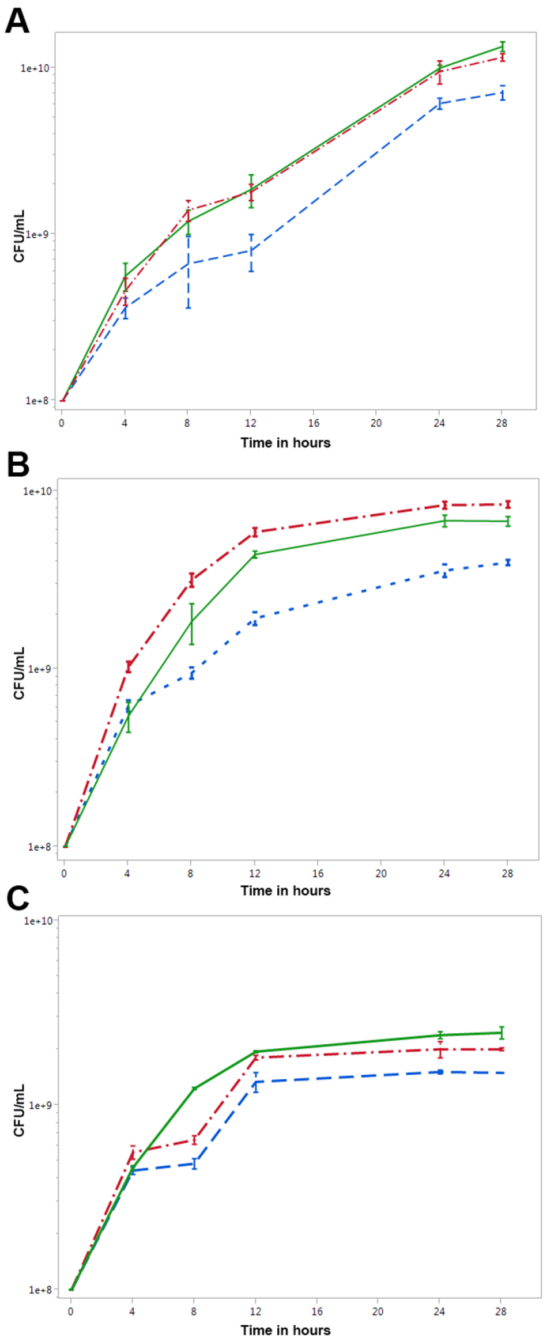

Figure A-2. Growth curves of human and bovine staphylococcal isolates Colony forming units (CFU/mL) of A) bovine *S. aureus* Newbould 305, B) human *S. aureus* USA300 and C) bovine *S. haemolyticus* grown in vitro for 28 hours in LB (green), SKM (red) and LIM (blue).

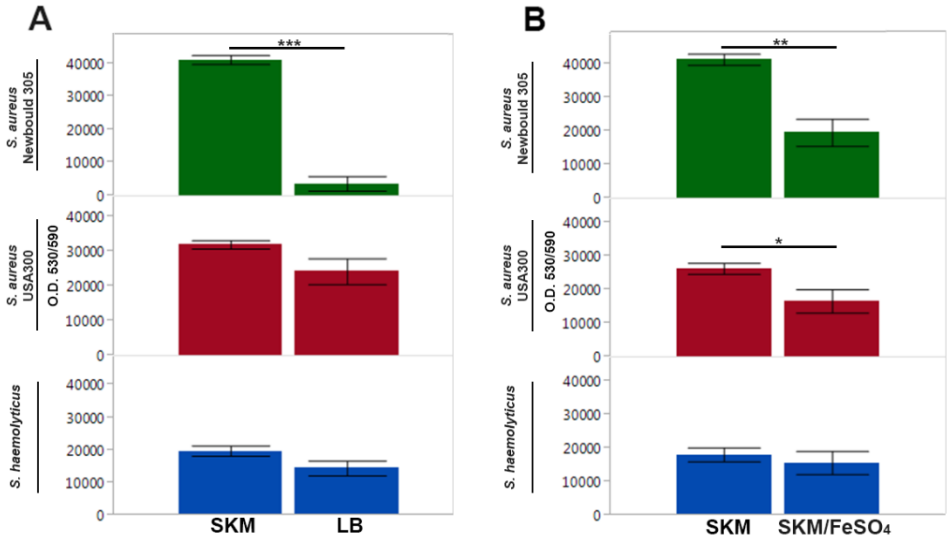

Figure A-3. Binding of human and bovine staphylococcal isolates to fibronectin. Fibronectin-binding as determined by metabolic dye assay of *S. aureus* Newbould 305, *S. aureus* USA300 and *S. haemolyticus* after growth in A) SKM versus LB and B) SKM versus SKM +FeSO4. Significance using the Student's T-test, $p \leq 0.05$(*), $p \leq 0.01$(**) or $p \leq 0.001$ (***), between fibronectin binding after growth in different media is shown.

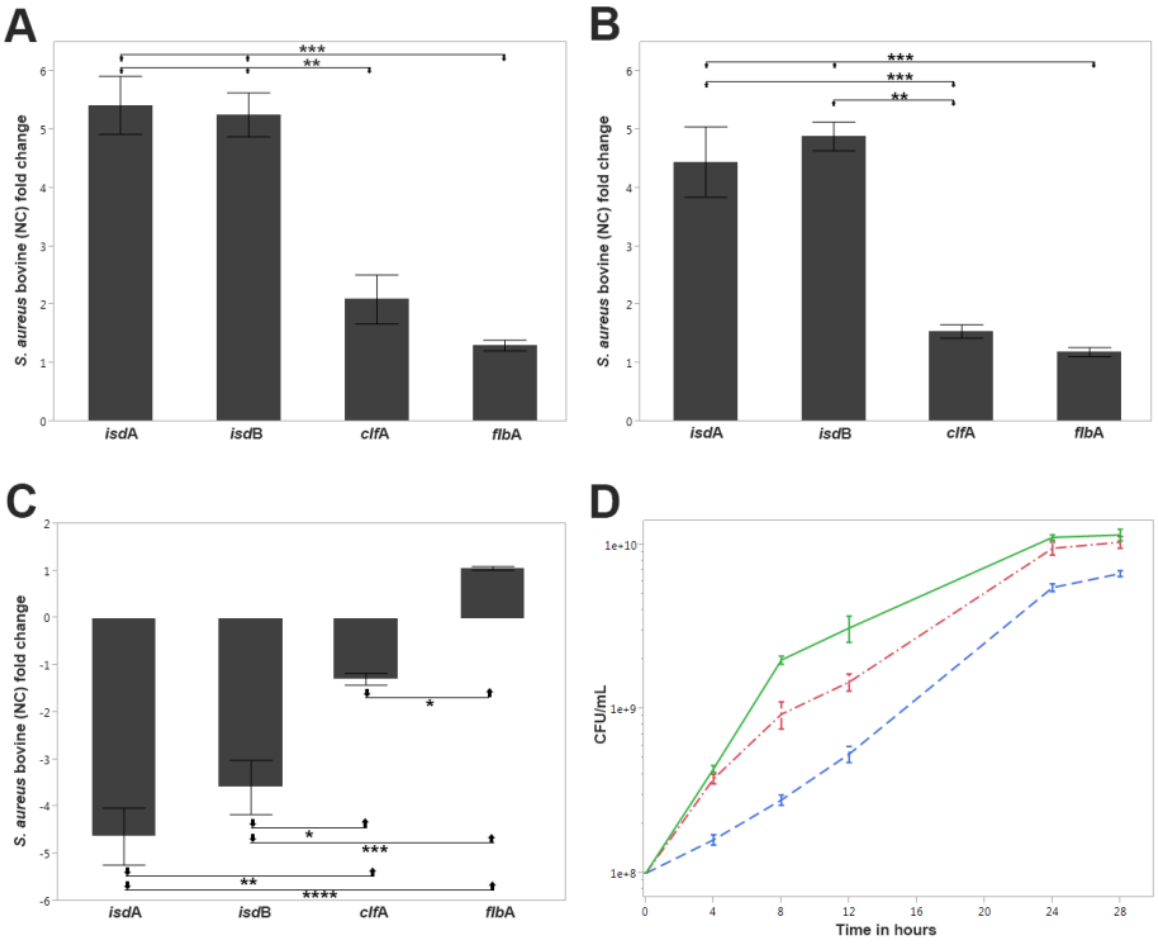

Figure A-4. Quantitative RT-PCR of adhesin expression from bovine *S. aureus* Novel Cow (NC) Normalized Ct values as fold change comparison of gene expression are shown after growth of NC in A) 5% skim milk (SKM) compared to Luria broth (LB), B) low iron media (LIM) compared to LB and C) SKM plus $FeSO_4$ compared to SKM alone, after 24 hours at 37°C. D) Growth curve in CFU/mL of NC growth LB (green), SKM (red) and LIM (blue). Significance using the Tukey-Kramer pairwise comparison, $p \leq 0.05$(*), $p \leq 0.01$(**), $p \leq 0.001$ (***) or $p \leq 0.0001$ (****) between the expression of genes is shown.