

**ROLE OF *STAPHYLOCOCCUS AUREUS* GapC AND  
GapB IN IMMUNITY AND PATHOGENESIS OF  
BOVINE MASTITIS**

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## ABSTRACT

Mastitis is the most prevalent and major cause of economic losses in dairy farms. Bovine mastitis caused by strains of *S. aureus* is a major economically important disease affecting the dairy industry worldwide. *S. aureus* is one of the most common udder pathogens that cause either clinical or sub-clinical mammary gland infections. Different treatment regimes have failed to cure *S. aureus* intramammary infections. Most mastitis vaccination strategies have focused on the enhancement of systemic humoral immunity rather than strengthening local intramammary immunity. Vaccines aimed at enhancing intramammary immunity of dairy cows against *S. aureus* mastitis have had limited success. Commercially available vaccines show various degrees of success and work in research laboratories with experimental vaccines suggest that in part, the failure of these vaccines lies in the limited antigenic repertoire contained in the vaccine formulations. Moreover, not only does variation in the antigenic composition but also presence of capsular polysaccharide in most pathogenic strains and decreased activity of immune effectors in milk affect the success of vaccines. In addition to these, the ability of *S. aureus* to attach and internalize into mammary epithelial cells, enables bacteria to escape from the effect of immunity and antibiotics by being hidden in the intracellular niche and thereby causing chronic recurrent intramammary infection. *S. aureus* also has the ability to become electron-transport-defective and to form slow-growing small colonies that are non haemolytic and less virulent. These small colony variants might hide from the immune surveillance in the intracellular area and revert to the parental strain causing chronic recurrent infections. If immunization targets antigenic molecules that are conserved throughout all pathogenic strains, even the small colony variants can be controlled since the immune system will clear the parental strain which causes lethal infection. Thus, immunization trials should focus on conserved immunogenic antigen molecules among pathogenic strains formulated with an adjuvant and delivered by a route of immunization to induce maximum stimulation of the immune system. Moreover, immunization should focus on inducing Th1 responses, which is protective against *S. aureus* mastitis. It has been reported that proteins with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity might be used as such antigens to induce protection against parasitic and microbial infections. Previous study in our laboratory on mastitis-causing streptococci indicates that GapC proteins of *S. uberis* and *S. dysgalactiae* have potential as vaccine antigens to protect dairy cows against mastitis caused by environmental streptococci. Two conserved cell wall associated proteins with

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, GapB and GapC have been identified from *S. aureus* isolates from bovine intramammary infections. The overall goal of this study was to improve our understanding on intramammary immunity using the GapC and GapB proteins of *S. aureus* as model antigens for mastitis and to determine the regulation of expression of *gapB* and *gapC* genes and their roles in the pathogenesis of bovine *S. aureus* mastitis. We hypothesized that strengthening local intramammary immunity using GapB and GapC proteins of *S. aureus* as antigens will protect against bovine *S. aureus* mastitis. To test this hypothesis we took the approach of using the *gapB* and *gapC* genes and constructed plasmids encoding GapB, GapC and GapB::GapC (GapC/B) chimeric proteins. We set six objectives to test our hypothesis using these proteins to enhance the intramammary immunity. In aim 1 we constructed plasmids encoding the GapB, GapC proteins and also constructed a chimeric gene encoding the GapC and GapB proteins as a single entity (GapC/B chimera) as the basis for a multivalent vaccine. In this objective the humoral and cellular immune responses to GapC/B were compared to the responses to the individual proteins alone or in combination in C57 BL/6 mice. Our results showed that the GapC/B protein elicited strong humoral and cellular immune responses as judged by the levels of total IgG, IgG1, IgG2a, IL-4 and IFN- $\gamma$  secretion and lymphocyte proliferation. These results strongly suggest the potential of this chimeric protein as a target for vaccine production to control mastitis caused by *S. aureus*. In aim 2 we continued our studies on GapC/B by testing the effects of DNA vaccination with plasmids encoding the individual *gapB* and *gapC* genes as well as the *gapC/B* protein gene with or without a boost with the recombinant proteins. The results showed that DNA vaccination alone was unable to elicit a significant humoral response and barely able to elicit a detectable cell-mediated response to the recombinant antigens but subsequent immunization with the proteins elicited an excellent response. In addition, we found that DNA vaccination using a plasmid encoding the GapC/B chimera followed by a boost with the same protein, although successful, is less effective than priming with plasmids encoding GapB or GapC followed by a boost with the individual antigens. In aim 3 we optimized immune responses in cows by comparing route of vaccination (subcutaneous versus intradermal), site of vaccination (locally at the area drained by the supramammary lymph node versus distantly at area drained by parotid lymph node). Our results showed that both subcutaneous and intradermal immunizations with the GapC/B protein at the area drained by the supramammary and parotid lymph nodes resulted in significantly increased serum and milk titers of total IgG, IgG1, IgG2,

and IgA in all vaccinated groups as compared to placebo. The anti-GapC/B IgG1 serum and milk titers were significantly higher in all vaccinated group as compared to the placebo group. These results indicated that vaccination at the area drained by the supramammary lymph node resulted in better immune responses. In aim 4 we tested different formulations of the GapC/B antigen with adjuvants such as PCPP, CpG, PCPP + CpG and VSA-3. We found that the VSA-3 formulation induced the best immune responses in cows. In this objective we also monitored immune responses longitudinally over one lactation cycle to determine the duration of immune responses by measuring IgG, IgG1, IgG2, and IgA on monthly blood and milk samples. We found that the duration of immune responses was about four months. In aim 5 we tested the role of GapC in the virulence of *S. aureus* mastitis using the *S. aureus* wild type strain RN6390 and its isogenic GapC mutant strain H330. Our results from both *in vitro* adhesion and invasion assays on MAC- T cells and *in vivo* infection of ovine mammary glands showed that GapC is an important virulence factor in *S. aureus* mastitis. In aim 6 we examined the role of *sar* and *agr* loci on the expression of *gapC* and *gapB* genes by qRT- PCR using *S. aureus* RN6390 and its isogenic mutants defective in *agrA*, *sarA* and *sar/agr* (double mutant) at exponential and stationary phases of growth. Our results showed that both *gapB* and *gapC* expression were down regulated in the mutant strains, indicating that the expression of the *gapB* and *gapC* genes is controlled by the universal virulence gene regulators, *agr* and *sar*. We also checked the role of environmental factors such as pH, growth media, and oxygen tension on the expression of *gapB* and *gapC* using q-RT-PCR. Our results showed that the expression of *gapB* and *gapC* genes in different strains of *S. aureus* was not consistent under the above-mentioned environmental conditions.

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## TABLE OF CONTENTS

PERMISSION TO USE POSTGRADUATE THESIS	i
ABSTRACT	ii
ACKNOWLEDGEMENTS	v
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS	xiii
1. LITERATURE REVIEW	1
1.1. Mastitis	1
1.2. Bovine <i>Staphylococcus aureus</i> Mastitis	2
1.2.1. <i>Staphylococcus aureus</i> Virulence Factors	3
1.2.1.1. <i>Staphylococcus aureus</i> cells Surface Proteins, Carbohydrates and Other Structural Components	4
1.2.1.2. Secreted Products	6
1.2.1.3. Adhesion of <i>Staphylococcus aureus</i> to Bovine Mammary Epithelial Cells	7
1.2.1.4. Invasion of <i>Staphylococcus aureus</i> into Bovine Mammary Epithelial Cells	7
1.2.1.5. Glyceraldehyde-3-phosphate dehydrogenase of <i>Staphylococcus aureus</i> as a Virulence Factor in Bovine Mastitis	8
1.2.1.6. Genetic Organization of <i>gapB</i> and <i>gapC</i> in <i>Staphylococcus aureus</i> .	10
1.2.1.7. Regulation of Virulence Factors in <i>Staphylococcus aureus</i>	12
1.2.1.8. Role of <i>agr</i> and <i>sar</i> in Biofilm Formation	17
1.3. Intramammary Defenses	18
1.3.1. Natural Defenses	18
1.3.2. Intramammary Immunity	18
1.3.2.1. Innate Immunity	18
1.3.2.2. Acquired Immunity	24
1.4. Treatment, Control and Prevention of Bovine <i>Staphylococcus aureus</i> Mastitis	33
1.4.1. Treatment of Bovine <i>Staphylococcus aureus</i> Mastitis	33
1.4.2. Control and Prevention of Bovine <i>Staphylococcus aureus</i> Mastitis	33
1.4.2.1. Vaccination	34
1.4.2.2. DNA Vaccines	37
2. HYPOTHESIS, OVERALL GOALS AND SPECIFIC OBJECTIVES	39

2.1. Hypothesis	39
2.2. Overall Goals and Rationale of this Study	39
2.3. Specific Objectives	40
3. MATERIALS AND METHODS	41
3.1. Bacterial Strains, Culture Media, Growth Conditions and Preparation of Competent Cells	41
3.1.1. Bacterial Strains and Growth Conditions	41
3.1.2. Preparation of Whey from Milk	42
3.1.3. Growth Conditions of <i>Staphylococcus aureus</i> Strains RN6390 and H330 for <i>in vitro</i> Adhesion and Invasion Assays and <i>in vivo</i> Challenge Trials	43
3.1.4. Infection	43
3.1.5. Determination of the Number of Total RN6390 and H330 Attached and Internalized into MAC-T Cells	44
3.1.6. Determination of the Number of Invasive RN6390 and H330	44
3.1.7. Preparation of <i>Staphylococcus aureus</i> Cells for Electroporation and Electroporation Protocol	44
3.1.8. Preparation of <i>Escherichia coli</i> for Electroporation and Electroporation Protocols	45
3.2. Culturing of Bovine Mammary Epithelial Cells	45
3.3. DNA Preparation, Plasmid Construction and Manipulation	46
3.3.1. Genomic DNA, PCR and Plasmid Purification Protocols	46
3.3.2. Construction of Plasmids Encoding GapC, GapB and GapC/B Used for DNA Immunizations.	46
3.3.3. Construction of Plasmids Encoding GapB, GapC and Chimeric GapC/B Recombinant Proteins for Expression and Purification in <i>Escherichia coli</i> .	47
3.3.4. Construction of a Plasmid Expressing the T-cell Plasminogen Activator Signal Sequence.	47
3.3.5. qRT-PCR	50
3.4. Purification of Proteins and Western Blots Analysis	51
3.4.1. Whole Cell Protein Extraction Procedure	51
3.4.2. Determination of Protein Concentration in the Whole Cell Lysate	52
3.4.3. Recombinant Protein Purification Procedure from <i>E. coli</i>	52
3.4.4. Western Blot	53
3.5. Transfection of MAC-T Cells and Immunohistochemistry	54
3.6. Mice Immunizations Protocols	54
3.6.1. Immunizations with Recombinant Proteins	54
3.6.2. Immunizations with DNA-Recombinant Proteins Combinations.	55
3.7. Optimizing Antibody Response in the Mammary Gland of Cows	56



3.8. Monitoring Duration of Immunity in Cows	57
3.9. Analysis of Humoral and Cellular Immune Responses	58
3.9.1. Enzyme-Linked Immuno-Sorbent Assay (ELISA)	58
3.9.2. ELISPOT and Lymphocyte Proliferation Assays	58
3.9.3. Peripheral Blood Mononuclear Cells Isolation and Assessment of Phagocytosis by Phagocytic cells.	59
3.10. Milk Sample Collection Procedure, Processing and Somatic Cells Count	60
3.10.1. Hygienic Milk Collection Procedure.	60
3.10.2. Somatic Cells Count	60
3.11. Statistics	60
4. EXPERIMENTAL RESULTS	61
4.1. Immune Responses to a GapC/B Chimera and Use as a Component of a Multivalent Vaccine against <i>Staphylococcus aureus</i> Mastitis	61
4.1.1. Introduction	61
4.1.2. Construction of the Chimeric <i>gapC/B</i> Gene	62
4.1.3. Humoral Immune Responses to the GapB, GapC and GapC/B Proteins	63
4.1.4. Cellular Immune Responses to the GapB, GapC and GapC/B Proteins	68
4.1.5. Antibody Recognition of Native GapC and GapB Proteins	70
4.1.6. Discussion	72
4.2. DNA-Protein Immunization against the GapB and GapC Proteins of a Mastitis Isolate of <i>Staphylococcus aureus</i>	76
4.2.1. Introduction	76
4.2.2. Construction of Plasmids Encoding a Protein Export Signal and Protein Expression on the Surface of MAC-T Cells	76
4.2.3. Immune Responses to DNA Vaccination	79
4.2.4. Immune Responses to DNA-Protein Vaccination	81
4.2.5. Cell-mediated Responses in Animals Boosted with the Recombinant Proteins	84
4.2.6. Discussion	88
4.3. Optimizing the Immune Response in the Mammary Gland of Cows.	92
4.3.1. Introduction	92
4.3.2. Humoral Immune Response in Serum and Mammary Gland of Cows	93
4.3.3. Discussion	96
4.4. Monitoring Duration of Immune Responses in Cows.	97
4.4.1. Introduction	97
4.4.2. Humoral Immune Responses in Serum and Milk of Cows.	99
4.4.3. Discussion	102
4.5. Role of GapC in the Virulence of Bovine <i>Staphylococcus aureus</i> Mastitis.	103
4.5.1. Introduction.	103

4.5.2. <i>In vitro</i> Adhesion and Invasion Assays of <i>Staphylococcus aureus</i> Strain RN6390 and its Isogenic GapC Mutant Strain H330 into Mammary Epithelial Cell Line (MAC-T)	103
4.5.3. Pilot Challenge Experiment Using the <i>Staphylococcus aureus</i> Strains RN6390 and its Isogenic GapC Mutant H330 on Ovine Mammary Gland	108
4.5.3.1. Clinical Findings, Milk Bacteriological Culture Results and Somatic Cell Counts	112
4.5.3.2. Gross Pathological and Histopathological Findings	115
4.5.4. Mastitis Challenge Trial with the <i>Staphylococcus aureus</i> Strain RN6390 and its Isogenic GapC Mutant Strain H330 on Ovine Mammary Gland (second trial)	120
4.5.4.1. Clinical Findings, Post-Challenge Milk Bacteriological Culture Results and Somatic Cell Counts	120
4.5.4.2. Gross Pathological and Histopathological Findings	121
4.5.5. Discussion	126
4.6. Regulation of Expression of <i>gapB</i> and <i>gapC</i> Genes.	127
4.6.1. Introduction	127
4.6.2. Expression of <i>gapC</i> and <i>gapB</i> Genes in Wild type (RN6390) and its Isogenic <i>agrA</i> , <i>sarA</i> and Double ( <i>sarA/agr</i> ) Mutant Strains	129
4.6.3. Expression of <i>gapB</i> and <i>gapC</i> Genes under Different Environmental Conditions	129
4.6.3.1. Expression of <i>gapB</i> and <i>gapC</i> Genes under Different Environmental Conditions during the Exponential Phase of Growth.	129
4.6.3.2. Expression of <i>gapB</i> and <i>gapC</i> Genes under Different Environmental Conditions during Stationary Phase of Growth.	131
4.6.4. Expression of GapB and GapC Proteins under Different Environmental Conditions	133
4.6.5. Discussion	141
5. CONCLUSIONS	143
6. REFERENCES	144

## LIST OF TABLES

Table 1.2.1.1. Summary of virulence factors of <i>Staphylococcus aureus</i> .....	11
Table 1.3.2.1. Receptors involved in innate immune recognition of pathogens.....	20
Table 1.3.2.2. Bovine antimicrobial peptides .....	23
Table 1.3.2.3. Regulatory T lymphocytes.....	28
Table 1.4.2.1. <i>Staphylococcus aureus</i> vaccines tested.....	36
Table 3.1.1.1. Bacterial strains and plasmids used in this study.....	42
Table 3.3.1. Synthetic oligonucleotides used in this study .....	49
Table 3.6.1.1. Vaccination of mice with recombinant proteins .....	55
Table 3.6.2.1. Immunization of mice with DNA-recombinant proteins combination .....	56
Table 3.7.1. Vaccination protocol for optimizing antibody response in the mammary gland of cows .....	57
Table 3.8.1. Vaccination protocol for monitoring duration of immune response in cows .....	57
Table 4.5.3.1 Pre-screening of animals for intramammary infection for pilot challenge trial ...	109
Table 4.5.3.2. Pre-screening of animals for intramammary infection for <i>in vivo</i> challenge trial	110
Table 4.5.3.3. Summary of pre- and post-challenge bacterial and somatic cell counts in both trials.....	111
Table 4.5.3.4. Peripheral blood mononuclear cells (PBMCs) count and number of intracellular bacteria after 24 hs of intramammary challenge.....	112
Table 4.6.2.1. Expression of <i>gapC</i> and <i>gapB</i> genes in wild type RN6390 and its isogenic <i>agrA</i> , <i>sarA</i> and double ( <i>sarA/agr</i> ) mutant strains.....	129
Table 4.6.3.1. Expression of <i>gapC</i> and <i>gapB</i> genes under different environmental conditions after 7 hs of incubation (Late exponential growth phase).....	131
Table 4.6.3.2. Expression of <i>gapC</i> and <i>gapB</i> genes under different environmental conditions after 12 hs of incubation (Stationary growth phase).....	133

## LIST OF FIGURES

Figure 1.2.1.7. Growth phase dependent regulation of virulence gene expression in <i>S. aureus</i> by <i>sar</i> and the <i>agr</i> loci. ....	16
Figure 4.1.2.1. Construction of a <i>S. aureus</i> GapC/B chimera. ....	63
Figure 4.1.3.1. Humoral total IgG, IgG1 and IgG2a titers against GapB, GapC and GapC/B....	66
Figure 4.1.3.2. Humoral immune responses to GapB, GapC and GapC/B.....	67
Figure 4.1.4.1. Cellular immune responses to GapB, GapC and GapC/B. ....	69
Figure 4.1.5.1A and B. Antibody recognition of GapC and GapB proteins.....	71
Figure 4.2.2.1. Construction of plasmids encoding protein export signal. ....	78
Figure 4.2.2.2. Expression of proteins on the surface of MAC-T cells. ....	79
Figure 4.2.3.1. Cellular immune responses to GapB, GapC and GapC/B after immunization with plasmid DNA. ....	80
Figure 4.2.4.1. Humoral immune responses to GapB, GapC and GapC/B after vaccination with plasmid DNA and boost with recombinant proteins. ....	83
Figure 4.2.5.1. Cellular immune responses to GapB, GapC, and GapC/B after vaccination with plasmid DNA and boost with recombinant proteins. ....	87
Figure 4.3.2.1. Serum titers to GapC/B. ....	94
Figure 4.3.2.2. Milk titers to GapC/B. ....	95
Figure 4.4.2.1. Duration of immune responses (Serum titers). ....	100
Figure 4.4.2.2. Duration of immune responses (Milk titers). ....	101
Figure 4.5.2.1. Number of RN6390 bacteria and its isogenic GapC mutant strain H330 attached and internalized into MAC- T cells at 37 °C.....	105
Figure 4.5.2.2. Number of RN6390 bacteria and its isogenic GapC mutant strain H330 attached and internalized into MAC- T cells at 4 °C.....	106
Figure 4.5.2.3. Number of intracellular bacteria recovered at different time points. ....	107
Figure 4.5.2.4. Number of intracellular bacteria released into supernatants after incubation in fresh media without antibiotics. ....	108
Figure 4.5.3.1a-c. Clinical udder examination results after 48 hs of intramammary challenge with RN6390 and its isogenic GapC mutant strain H330.....	115
Figure 4.5.3.2a-c. Gross pathological changes in the right side udder halves that received wild type strain.....	116
Figure 4.5.3.3a-c. Haematoxylin and eosin stained mammary gland tissue experimentally challenged with GapC mutant strain H330.....	117
Figure 4.5.3.3d-f. Haematoxylin and eosin stained mammary gland tissue experimentally challenged by wild type <i>S. aureus</i> strain RN6390.....	118
Figure 4.5.3.4a-c. Gram-stained mammary gland tissue from wild type strain RN6390 challenged udders.....	119
Figure 4.5.4.2. Gross pathological findings in udder halves challenged by wild type RN6390 (a and b) and mutant strain H330 (c and d).....	121
Figure 4.5.4.3a-d. Hematoxylin and eosin stained udder tissue from wild type strain RN6390 challenged Udders.....	123

Figure 4.5.4.4a and b. Hematoxylin and eosin stained udder tissue from the mutant strain H330 challenged udder. ....	124
Figure 4.5.4.5a-c. Gram-stained gland tissue from wild type strain RN6390 and mutant strain H330 challenged udder. ....	125
Figure 4.6.4.1a-f. GapB and GapC proteins from the supernatants of cells grown in different media. ....	135
Figure 4.6.4.2a–e. GapB and GapC proteins in the whole cell lysate grown in different media. ....	138

## LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
<i>Agr</i>	Accessory gene regulator
AIP	Autoinducing peptide
AP	Alkaline phosphatase
APC	Antigen presenting cells.
BCIP	5-bromo-4-chloro-3-indol phosphate
BSA	Bovine serum albumin
CBDN	Canadian Bacterial Disease Research Network
CBMRN	Canadian Bovine Mastitis Research Network
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
ClfA	Clumping factor A
ClfB	Clumping factor B
CpG	Cytosine linked to a guanine by a phosphate bond
CR1	Complement receptor 1
CR3	Complement receptor 3
CSF	Colony stimulating factor
CTLA-4	Cytotoxic T- lymphocyte associated antigen-4
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot
Fc $\gamma$ R	Constant fragment gamma receptor
FnBPA	Fibronectin binding protein A
FnBPB	Fibronectin binding protein B
GapB	Glyceraldehyde-3-phosphate dehydrogenase B

GapC	Glyceraldehyde-3-phosphate dehydrogenase C
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	Group A streptococci
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
Hep2 cells	Cell line established from human laryngeal carcinoma
<i>Hld</i>	Delta hemolysin
IFN- $\gamma$	Interferon gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
KDa	Kilodalton
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Mac-1	Receptor for complement fragment C3bi (iC3b), present on neutrophils and mononuclear phagocytes or A $\beta_2$ integrin.
MAC-T	Transformed mammary epithelial cells.
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
NI-NTA	Nickel-nitrilotriacetic acid
NK	Natural killer
NSERC	Natural Sciences and Engineering Research Council of Canada.
ORF	Open reading frame
PBS	Phosphate buffered saline
PCPP	Poly [di (carboxylatophenoxy) phosphazene.
PCR	Polymerase chain reaction
PMN	Polymorphonuclear neutrophils
PNSG	Poly-N-succinyl B-1-6 glucosamine

q-RT PCR	Quantitative reverse transcriptase polymerase chain reaction
RAP	RNAIII activating protein
RNA	Ribonucleic acid
RNAIII	Ribonucleic acid III
SA	<i>Staphylococcus aureus</i>
<i>Sar</i>	Staphylococcal accessory regulator
SCC	Somatic cell counts
SDS	Sodium dodecyl sulphate
SEC1	<i>Staphylococcus aureus</i> enterotoxin-C1
TBS	Tris buffered saline
Th	T helper
TLR	Toll-like receptor
TMG	Tris minimal glucose
TMS	Tris minimal succinate
TNF- $\alpha$	Tumor necrosis factor alpha
TRAP	Target of RNAIII activating protein
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VIDO	Vaccine and Infectious Disease Organization
VISA	Vancomycin intermediate resistant <i>Staphylococcus aureus</i>



# 1. LITERATURE REVIEW

## 1.1. Mastitis

Mastitis is defined as an inflammation of the mammary gland which is characterized by redness, heat, swelling and pain. Mastitis is often expressed as an increased somatic cell count in the milk with isolation of potentially mastitis causing pathogens [1, 2]. Microorganisms, irritant chemicals and physical or mechanical damage can cause inflammation of a mammary gland. Although the impact of mammary gland inflammation by chemicals and physical or mechanical damage on dairy productivity is not known, almost all cases of mastitis in dairy farming are believed to be caused by pathogenic microorganisms. In dairy cows the cause is nearly always pathogenic microorganisms [3], usually contagious (*S. aureus*, *S. agalactiae*, *Mycoplasma* and *Arcanobacterium*) and environmental (coliform bacteria group and the environmental streptococci) [4-6] bacteria. The main reservoirs of infection for contagious and environmental mammary pathogens are infected quarters and the surrounding environment of dairy cows, respectively. The genetic selection of cows for milk production has increased milk productivity but decreased resistance to mastitis. This decreased resistance to mastitis is believed to be due to metabolic stresses associated with milk production [7-9]. Improper use of milking machines, intensive indoor housing, and use of bedding materials that support bacterial growth also have a significant impact on predisposing dairy cows to mastitis [10]. Some of the factors known to affect the incidence and prevalence of mastitis are lactation stage, parity number, damage to the skin of the udder, and season [11-13]. The complex interaction between the intramammary pathogens, mammary gland immune effectors and environmental factors that play a role in the establishment of intramammary infection as well as protective intramammary immunity should be clearly defined for an appropriate immunization strategy to be implemented.

Infection of the mammary gland by pathogenic microorganisms results in either clinical or subclinical mastitis. Clinical mastitis is characterized by visible inflammatory changes in the mammary gland and changes in milk quality and composition. These changes can be detected by physical clinical examination. Bacteriological culture of milk from clinical mastitis is also important to identify causative agents. However, bacteriological milk culturing should be done early in the development of mastitis since some culture results might be negative since the causal

agent could be removed by the host immune system. Local clinical signs of mastitis include a swollen udder with hard consistency, changes in milk colour and consistency, and a painful udder. Systemic signs of clinical mastitis include increased body temperature, depression, and loss of appetite and weight while in subclinical mastitis, there are no grossly visible inflammatory changes in both gland tissue and milk. Inflammation can only be detected by the presence of increased numbers of somatic cells in the milk.

Mastitis is a major cause of economic losses in dairy farming [14-16]. These losses are primarily due to reduced milk yield [15, 17], rapid spoilage of mastitic milk and the discarding of milk with antibiotics [18], treatment and replacement costs [19, 20], lower price of poor quality milk [20, 21], increased culling rate or death from infection [22, 23], and decreased fertility [24, 25]. Mastitis also affects public health due to food spoilage by pathogenic microorganisms in mastitic milk [26-28] and production of toxins by these microorganisms [29, 30]. Some potential mastitis pathogens such as *Mycobacterium*, *Brucella*, and *Leptospira* and *Listeria* species are zoonotic microorganisms that can infect humans from dairy products [31]. These pathogens can be shed through milk either from a primary gland infection or as secondary events to other systemic infections in the body. Frequent antibiotic treatment of mastitic cows may lead to increased prevalence of antibiotic resistance in animal pathogens and subsequent transfer of bacteria or a resistance gene to human pathogens [32-34].

## **1.2. Bovine *Staphylococcus aureus* Mastitis**

Taxonomically, *S. aureus* belongs to the family of *Staphylococcaceae* and genus *Staphylococcus*. It is Gram-positive, catalase and coagulase positive, nonspore forming, oxidase negative, nonmotile, cluster-forming, facultative anaerobe that grows by aerobic respiration or by fermentation that yields lactic acid. It can ferment mannitol which is one of the tests to distinguish *S. aureus* from *S. epidermidis*. In spite of the fact that the coagulase test is not accurate for the identification of *S. aureus*, more than 95% of all coagulase positive staphylococci from bovine mastitis belong to *S. aureus* [35]. Other coagulase-positive strains are *S. hyicus* and *S. intermedius*. *S. aureus* can grow at a temperature range of 15 to 45 °C and at NaCl concentrations as high as 15%. It forms golden yellow colonies on solid growth media.

*S. aureus* is one of the most frequently isolated contagious mastitis pathogens that cause either clinical or subclinical mammary gland infection [36, 37]. The prevalence of *S. aureus* mastitis could increase from < 5% to more than 30% in a year, causing significant increases in milk somatic cell count [38]. Multiple strains can be involved in causing mastitis in a single herd or in different herds from a similar or a different geographical area [39, 40]. An individual cow [40, 41] or quarter of the same udder [42] may be infected simultaneously by more than one strain of *S. aureus*. However, in different countries only clones that have a wide geographic distribution are responsible for most of the cases of bovine intramammary infections [43]. Studies to determine the diversity of *S. aureus* colonizing cattle and humans indicated that the strains prevalent in humans were not similar to the causative agents of bovine mastitis [44]. The virulence potential does not differ significantly between human and animal isolates of *S. aureus*; however, *S. aureus* that causes mastitis in farm animals represent a separate genetic cluster dominated by the presence of the toxic shock syndrome toxin encoding gene [45]. The main reservoirs of *S. aureus* are infected quarters and the skin of the udder and the teat [46]. The molecular epidemiological analysis of *S. aureus* strains from diverse clinical and geographical origins using pulsed-field gel electrophoresis and binary typing indicated an association between distinct genotypes and severity of mastitis [40].

### **1.2.1. *Staphylococcus aureus* Virulence Factors**

*S. aureus* colonizes the teat end and it moves to the intramammary area either by progressive colonization or by the changes in intramammary pressure, especially at the end of milking [36]. In the intramammary area it can adhere to epithelial cells, multiply and colonize the tissue [36, 47, 48]. The pathogenicity of *S. aureus* is multifactorial and most staphylococcal diseases are due to many combined factors rather than a single factor. The *S. aureus* virulence factors comprise cell surface structural components such as proteins, lipids, carbohydrates, proteoglycans, glycolipids and secretory products. Based on their biological activities, *S. aureus* virulence factors can be divided into three general functional categories: those that mediate adhesion of bacteria to host cells or tissue (adhesins), those that promote tissue damage and spread (invasins) and those that protect the bacteria from the host immune system. Thus, the

pathogenicity of *S. aureus* depends on the combined action of cell surface structural components, different extracellular toxins and enzymes [49]. The expression of virulence factors of *S. aureus* is dependent on the bacterial growth phase. The cell surface components are expressed during exponential growth phase whereas the secretory factors are expressed during post exponential growth phase [50-54] (Fig. 1.2.1.7).

### **1.2.1.1. *Staphylococcus aureus* cells Surface Proteins, Carbohydrates and Other Structural Components**

#### **i. Protein A**

The cell surface of *S. aureus* comprises the cell wall, the underlying cell membrane and in most pathogenic strains the outer layers of exo polysaccharides [55, 56]. Protein A is distributed throughout the cell wall in most virulent strains of *S. aureus* isolated from bovine intramammary infections [57] and is believed to have antiphagocytic activity [56-58]. Protein A binds to the Fc portion of IgG antibody and prevents the action of anti-staphylococcal activity [59]. Structurally, protein A is linked to the cell wall and the N-terminal domain which contains five IgG binding repeats is exposed on the bacterial cell surface [60, 61]. The five N-terminal repeat domain binds to CH<sub>2</sub> and CH<sub>3</sub> segments of the Fc portion of IgG [61, 62] which is at the C $\gamma$ 2-C $\gamma$ 3 domain interface region [63, 64]. Staphylococcal protein A also binds to the heavy chain variable region of Fab fragment of some monoclonal and polyclonal IgM, IgG, IgA and IgE proteins and this site is believed to be different from the antigen-binding site because the Fab $\gamma$  fragment of mouse monoclonal antibody was found to bind both protein A and antigen at the same time [65].

*S. aureus* also expresses many other proteins including extracellular adherence protein (Eap) [66-68], fibronectin binding proteins (FnBPs) [68-70], collagen binding protein [71], fibrinogen binding proteins (FgBP) [72, 73], a vitronectin binding protein [66], and elastin binding protein [74]. These surface proteins serve as adhesins to allow the bacterium to attach to the host cell surface during early stage of infection to achieve colonization of host tissue (Table 1.2.1.1).

## **ii. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)**

Two conserved proteins that have glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity have been identified in *S. aureus* including bovine clinical mastitis isolates. These proteins were named GapB and GapC [75]. Glyceraldehyde-3-phosphate dehydrogenase is a known key glycolytic enzyme in both prokaryotic and eukaryotic organisms that reversibly catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate into 1, 3, bi-phosphoglycerate. Results from recent studies revealed that this enzyme is localized on the cell surface of many pathogenic microorganisms [75-79] and it binds to cellular molecules such as plasmin, transferrin, myosin and actin [75, 78-82]. In streptococci, surface located proteins with GAPDH activities have been shown to have pathogenic and immunostimulatory roles. Some of these proteins induce post-translational modification of eukaryotic proteins including phosphorylation and ADP ribosylation [83]. Glyceraldehyde-3-phosphate dehydrogenase also serves as a ligand (adhesin) on the surface of pathogenic microorganisms that binds to specific receptor molecules on the surface of host cells [84, 85].

## **iii. Exopolysaccharides**

One of the *S. aureus* cell surface structural components that have a role in virulence is capsular polysaccharide. Capsular polysaccharide is an exopolysaccharide layer that covers the cell wall of some strains including most clinical isolates of *S. aureus* from human infections and bovine mastitis [86-90]. There are 11 capsular polysaccharide serotypes in *S. aureus* isolates from human infection whereas 12 serotypes had been identified in strains from bovine mastitis. Serotypes 1 and 2 are heavily encapsulated and form mucoid colonies on solid media whereas serotypes 3–12 are microencapsulated and form non-mucoid colonies on solid media. Strains of serotypes 5 and 8 are frequent isolates from bovine mastitis as is the case with human endovascular infection [55, 56, 86, 87]. The capsular polysaccharide does not impede deposition of antibodies on the highly antigenic epitopes on cell wall; however, it masks recognition of antibody by neutrophils and other phagocytic cells [56, 57, 91, 92]. However, if antibody is

produced against capsule itself, for example by attaching capsule to immunogenic carrier molecule (haptin), it serves as effective opsonins for neutrophils [93, 94]

Slime is an exopolysaccharide component loosely bound to the bacterial surface [56, 91, 95]. It has the characteristic feature of enabling bacteria to form microcolonies [91, 95] which may allow bacteria to evade host defenses and the effects of antimicrobials. Slime production is strain dependent and phase variation in slime production is observed [91, 96].

Teichoic acids are a group of polymers in the cell-wall, capsule, or membrane of Gram-positive bacteria that contain glycerol phosphate or ribitol phosphate residues [97]. There are differences between cell-wall linked teichoic acids and plasma membrane linked teichoic acids. Cell wall teichoic acids are covalently attached to peptidoglycan that may show glycerol phosphate- and ribitol phosphate-containing structures. They are not present in all species of Gram-positive bacteria and their presence depends on growth conditions [98]. In contrast, membrane teichoic acids are covalently bound to a glycolipid moiety of the plasma membrane. They are more relevant structures than cell-wall teichoic acids and their presence is not dependent on growth conditions [98]. The covalent attachment of membrane glycerol teichoic acids to glycolipid led to the naming as lipoteichoic acid for these polymers [99]. Teichoic acid and lipoteichoic acid are reported to serve as receptors for adhesion to epithelial cells of mucosal surfaces [100] and are also reported to have role in allowing bacteria to resist host defense peptides [101]. Their exact role in bovine *S. aureus* mastitis is not known.

#### **1.2.1.2. Secreted Products**

Haemolytic toxins (mainly  $\alpha$  and  $\beta$  toxins) play a role in the pathogenesis of bovine *S. aureus* mastitis [57, 102-105]. Alpha toxin binds to the plasma membrane of the epithelial cell and oligomerises into hollow hexameric complexes, which form transmembrane pores. The transmembrane pores result in the leakage of low molecular weight molecules from the cytosol and lead to cell death [106, 107]. Beta toxin is a sphingomyelinase C that hydrolyses the

sphingomyelin present in the exoplasmic leaflet of the plasma membrane resulting in increased permeability [57] with a rapid efflux of K<sup>+</sup> and influx of Na<sup>+</sup>, Cl<sup>-</sup> and Ca<sup>2+</sup> [106] leading to lysis of cells.

Staphylococcal enterotoxins are extracellular proteins mainly composed of a single polypeptide chain [43]. There are many serologic types of these toxins, the major ones include A, B, C1, C2, C3, D, E, and H [43, 108]. Some of the enterotoxins are known to bind to MHC molecules at different site from the peptide binding groove which results in T cell activation and cytokine production [109]. This over-stimulation by superantigens leads to systemic shock and disseminated intravascular coagulation (DIC) similar to lipopolysaccharides (LPS) of Gram-negative bacteria. Leukocidin toxin has cytolytic effects on bovine polymorphonuclear neutrophils and macrophages [110]. Some of the *S. aureus* strains from intramammary infection have the ability to produce the toxic shock syndrome toxin-1 [111] which causes toxic shock due to its superantigenic activity. *S. aureus* also produces numerous extracellular enzymes which have been implicated in the pathogenesis of bovine intramammary infection and human infections [49] (Table 1.2.1.1).

#### **1.2.1.3. Adhesion of *Staphylococcus aureus* to Bovine Mammary Epithelial Cells**

*S. aureus* is one of the most important causative agents of bovine mastitis that is shown to adhere to bovine mammary gland cells *in vivo* [112-114] and *in vitro* [113, 115-122]. *In vitro* adhesion varies with *S. aureus* strains, growth medium, growth phase, and the origin of mammary epithelial cells. The kinetics of *S. aureus* adhesion to mammary epithelial cells is not well characterized. Adhesion may be achieved through non-specific physiochemical and/or specific interactions between a ligand, associated with the bacterial cell surface and a receptor on the host cell surface.

#### **1.2.1.4. Invasion of *Staphylococcus aureus* into Bovine Mammary Epithelial Cells**

Intracellular *S. aureus* have been demonstrated in mammary secretory epithelial cells of experimentally infected mice [123], cultured mammary epithelial cells from cows [113, 115, 116, 124] and epithelial cells isolated from mastitic milk ([125]). *In vitro* studies have also shown

that *S. aureus* also invades other non-phagocytic cells such as endothelial, fibroblast and osteoblast cells [126-130]. Invasion may occur in a bacterium-specific endocytic process that involves the participation of elements of the cytoskeleton and/or through another receptor-mediated energy-requiring mechanism that involves both eukaryotic and prokaryotic cellular functions like *de novo* protein synthesis [115]. Many bacterial cell surface adhesins seems to be involved in adhesion and invasion of mammary epithelial cells [68, 131-134]. *S. aureus* has also been shown to induce apoptosis after being internalized into mammary epithelial cells [135, 136].

#### **1.2.1.5. Glyceraldehyde-3-phosphate dehydrogenase of *Staphylococcus aureus* as a Virulence Factor in Bovine Mastitis**

Glyceraldehyde-3-phosphate dehydrogenase is involved in the microbial energy metabolism from a glucose source [137, 138] and it catalyzes oxidative phosphorylation of D-glyceraldehyde-3-phosphate (G3P) to 1, 3-diphosphoglycerate in the presence of phosphate, and NAD<sup>+</sup> as follows:



Studies in the past two decades have demonstrated the presence of GAPDH with non enzymatic functions on the cell surface of some pathogenic microorganisms. The GAPDH of Group A streptococci (*S. pyogenes*) binds to plasmin, fibronectin, lysozyme, myosin and actin [78, 139] and it also acts as an ADP-ribosylating enzyme [83, 140]. *Streptococcus gordonii*, which causes endocarditis, highly expressed GAPDH on its surface at pH 6.5 [141]. Glyceraldehyde-3-phosphate dehydrogenase of mycobacteria had been shown to bind to epidermal growth factor [142]. Enteropathogenic *E. coli* (EPEC) was shown to secrete GAPDH as one of the proteins to induce signal transduction events in epithelial cells [143]. The expression of GapC of *E. coli* was shown to increase in the natural environment as compared to enriched growth media [144]. It had also been reported recently that association with host cells increased the expression of the *gap* genes in *Neisseria meningitidis* and *N. lactamica* with concomitantly increased expression and surface deposition of the corresponding protein [145]. In bovine clinical mastitis isolates of *S. aureus*, two proteins with GAPDH activity (GapC and GapB) have been identified [75]. These proteins bind to transferrin and plasmin [75]. These authors also showed that the GapC protein was present on the surface of all *S. aureus* strains tested. In human isolates of *S. aureus* and *S.*



*epidermidis* a 42 kDa protein with GAPDH activity was found on the cell surface [79, 81]. In fungal pathogens such as *Candida albicans*, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, GAPDH had been shown to be located on the cell surface [76, 146]. GAPDH of *Candida albicans* has been shown to bind to fibronectin and laminin [80].

Recent evidence confirmed that GAPDH is involved in the interaction between pathogenic microbes and host cells. In *S. pyogenes*, SDH (GAPDH) binds to Detroit human pharyngeal cells surface-exposed urokinase plasminogen-activator receptor/CD87 [84]. This protein was shown to induce apoptosis [147] and to be involved in immune evasion by inhibiting C5a-dependent chemotaxis and H<sub>2</sub>O<sub>2</sub> production [148, 149]. A streptococcal SDH (GAPDH) mutant strain of *S. pyogenes*, which could not secrete SDH out of the cell, decreased its binding capability to plasminogen and human pharyngeal cells and also lost its immune evasion effect [150]. The prevention of cell surface export of SDH affects the pathogenicity of *S. pyogenes*, confirming that SDH is an important virulence factor [150]. Moreover, very recently in *S. agalactiae* GAPDH was found to be a pathogenicity related protein [151]. These authors also showed that the enzymatically active recombinant GAPDH protein stimulated expression of CD69 on B cells from mice. They also showed an increased number of IgG secreting cells in spleen and that a SDH (GAPDH) over-expressing strain is more virulent than the wild type strain and these effects were IL-10 dependent. In another study, *in vitro* adherence assay data demonstrated a significant reduction in adhesion of *Streptococcus suis* strain 2 (SS2) to HEp-2 cells pre-incubated with purified GAPDH compared to non pre-incubated controls, indicating that the GAPDH mediates SS2 bacterial adhesion to host cells [85]. The GAPDH of *Streptococcus oralis* binds to the fimbriae of *Porphyromonas gingivalis* and leads to *P. gingivalis* colonization of periodontal sites [152]. In *Mycoplasma genitalium* GAPDH serves as an adhesin that bind to mucin for adherence and colonization of host tissue [153]. The secretion of GapC of enteropathogenic *E. coli* as a result of bacterial interactions with host cells [143, 144] and the increased expression of a GAPDH gene and its protein deposition on the cell surface of *Neisseria* species [145] shows the involvement of this protein in the pathogenesis of these organisms. Moreover, the GAPDH of *Leishmania infantum* amastigotes allows the parasite to resist nitric oxide produced by host phagocytic cells [154]. More importantly, it has been shown that human microflora *Lactobacillus plantarum* LA 318 adheres to human colonic mucin using

its surface GAPDH which might be the case for several other microflora in diverse host species[155]. These observations indicated that GAPDH is an adhesin for colonization of tissue surfaces including mammary gland both for pathogenic and non-pathogenic normal microflora. Based on role of GAPDH in other microorganisms, it is logical to postulate that the staphylococcal GAPDH by the virtue of its surface localization may function as an adhesin for *S. aureus* colonization of host tissue surfaces including the bovine mammary gland.

#### **1.2.1.6. Genetic Organization of *gapB* and *gapC* in *Staphylococcus aureus*.**

In *S. aureus* the *gapB* and *gapC* genes are located in two different regions of the chromosome [156, 157]. Immediately downstream of the *gapC* gene there are genes encoding the glycolytic enzymes phosphoglycerate kinase (*pgk*), triosephosphate isomerase (*tpi*), 2, 3-diphosphoglycerate-independent phosphoglycerate mutase (*pgm*) and enolase (*eno*). Immediately upstream of *gapC* is *gapR*, known to be a regulator of this operon in *Streptomyces aureofaciens* [158]. The presence of a gene located upstream of *gapC* with high homology to *gapR* of the human strains suggests a similarity in the genetic organization of *gapC* of bovine and human isolates; however, this needs to be determined.

Table 1.2.1.1. Summary of virulence factors of *Staphylococcus aureus*

<b>Virulence factor</b>	<b>Effects</b>	<b>Reference</b>
<b>Cell surface factors</b>		
FnBPA and FnBPB	Attachment to fibronectin	[159]
ClfA and ClfB	Adhesion to fibrinogen	[160]
Protein A	Immune evasion	[51, 161-163]
GAPDH	Adhesion to transferrin/plasmin	[81]
Collagen binding protein	Adhesion to collagen	[164]
Elastin binding protein	Binding to elastin	[165]
MHC analogous protein	Binding to extracellular matrix proteins	[68, 166 , 167]
Polysaccharide intercellular adhesin	Intercellular adhesion and biofilm formation	[165]
Coagulase	Binding to fibrinogen	[161, 162, 168, 169]
Capsular polysaccharides (type I, 5, and 8)	Antiphagocytic molecule	[170, 171]
Polysaccharide intercellular adhesin and biofilm associated protein	Prevent adhesion and invasion, decrease phagocytic ingestion and killing	[172-174]
Teichoic acid	Protection against human and animal host defense peptides and also adhesin	[101]
Lipoteichoic acid	Induce acute phase proteins, and also adhesin	[175, 176]
<b>Extracellular factors</b>		
Staphylococcal Enterotoxins: A – E, H	Evasion of host defences with superantigen function, Food associated diarrhea	[177-179]
Toxic shock syndrome toxin-1	Evasion of host defenses with superantigen properties, cause TSS	[51, 178]
Exfoliative toxins A and B	Evasion of host defenses, cause staphylococcal scalded skin syndrome	[165]
Lipase	Evasion of host defense	[180]
V8 protease	Tissue invasion and modification of surface proteins	[49, 178, 181]
Serine proteases	Degrade tissue enzymes	[162]
Zinc-metalloproteinase (auriolysin)	Processing enzyme?	[49, 181]
$\beta$ - lactamase	Resistance to $\beta$ - lactam -type antibiotics	[182]
Methicillin-resistance surface protein	Resistance to methicillin	[165]
Panton-Valentine leukocidin	Evasion of host defenses, lysis of phagocytes	[162, 178]
Staphylokinase	Plasminogen activator	[51]
$\alpha$ -hemolysin	Tissue invasion, form pores in host cell membrane	[51, 162, 178, 183]
$\beta$ - hemolysin	Tissue invasion, sphingomyelinase	[51 , 161 , 162]
$\delta$ - hemolysin	Potentialiation of $\beta$ - hemolysin	[51 , 75 , 183]
$\gamma$ -hemolysin	Potentialiation of host cell lysis	[162 , 184]
Phospholipase C	Lysis of host cells	[49]
Elastase	Tissue invasion	[182]
Hyaluronidase	Tissue invasion	[161 , 165, 182 ]

### 1.2.1.7. Regulation of Virulence Factors in *Staphylococcus aureus*

Environmental stimuli lead to various changes in microorganisms and these stimuli induce changes in cellular activity by inducing transcription of specific genes that are required to adapt to changes in the environment. In bacteria, stimuli can be external from the environment or internal from their own products. The bacterial products serve as stimuli to induce transcription of certain sets of genes which help the whole population of bacterial cells to synchronize their activities to adapt to changes at a population level. These products provide mechanisms for cell to cell cross talk in a cell quantity dependent manner (quorum-sensing) to better adapt the whole population of bacteria to the new changes or stimuli [185-187].

The pathogenicity of *S. aureus* is due to the combined action of several cell surface structural components and secreted extracellular enzymes and toxins [185-187]. The expression of multiple virulence factors of *S. aureus* is controlled by different global regulatory genes such as staphylococcal accessory regulator (*sar*) [52, 188], and two component regulatory systems [49, 165, 189]. There are 16 two component regulatory systems in the *S. aureus* genome and some of these include: accessory gene regulator (*agr*) [190], *S. aureus* exoprotein expression (*saePQRS*) locus [191], staphylococcal respiratory response (*srrAB*) locus [192], autolysis related locus (*arlSR*) [193] and *lytRS* systems [194, 195]. Moreover, the alternative sigma factor (*sigB*) [196] is also involved in the regulation of several virulence factors in *S. aureus*. These global regulatory genes encode many proteins that are involved in the regulation of expression of several *S. aureus* cell surface and extracellular virulence factors in a growth-phase dependent manner [185-187](Figure 1.2.1.7).

Staphylococcal accessory regulator A (SarA)[188] and its homologues SarR [197], Rot [198], SarS [183], SarT[199] and SarU [200] are DNA binding proteins that are known to regulate the expression of several cell surface and extracellular virulence genes in *S. aureus*. The *sarA* operon consists of three overlapping transcripts with similar 3' end that are under the control of three different promoters *sarP1*, *sarP3* and *sarP2* (Fig. 1.2.1.7). All three transcripts encode the 14.5 kDa SarA protein [186, 201]. The production of SarA depends on bacterial

growth phase and maximum production is achieved at late exponential phase. The SarA protein binds to the promoter region of *agr* and induces *agr* activation and it binds to specific sequences in the promoter region of *S. aureus* cell surface protein genes and regulates their expression. The increased production of SarA during post exponential phase [197] is matched with increased expression of *agr* [202]. The *sarA* locus increases the production of cell surface components during early logarithmic phase as well as that of  $\alpha$ -,  $\beta$ -, and  $\delta$ -toxins during late exponential phase [190, 203].

The *sarR* encodes a 13.6 kDa protein which has homology with some sequences of SarA. The expression of SarR peaks at post exponential growth phase and it induces down regulation of SarA [186, 197]. The SarS protein was originally identified as SarH1 [183] and later as SarS [204]. The *sarS* gene is transcribed from two different promoters and SarS up-regulates expression of staphylococcal protein A (*spa*) and down-regulates the expression of alpha toxin. Staphylococcal accessory regulator S (SarS) expression is down regulated by both *sar* and *agr* [183, 186]. The SarT is a 16.1 kDa protein known to down-regulate the expression of *hla* and is down regulated by either Agr or SarA [199]. Staphylococcal accessory regulator U (SarU) is a 29.3 kDa protein believed to be involved in the *agr* system, is down-regulated by SarT [200], and mutation of *sarU* decreases expression of *agr* activated genes. The Rot (repressor of toxin) is a 15.6 kDa protein [198] reported to control regulation of several genes in an opposite manner to *agr*. Rot is believed to be involved in up-regulation of cell surface proteins required for early colonization of host tissue and down-regulation of the expression of extracellular toxins and proteases during post exponential phase [162]. There are other members of SarA homologues such as SarY (29.8 kDa), SarV (13.9 kDa), SarX (16.7 kDa). Staphylococcal accessor regulator-Y (SarY) is reported to be similar to SarR and SarT whereas SarV and SarX are similar to Rot [165, 186].

The *agr* locus is one of the two component systems of *S. aureus* that controls the expression of virulence factors in a bacterial growth-phase dependent manner. The *agr* gene up-regulates the expression of secreted toxins during post exponential growth phase and down regulates the expression of cell surface components during the logarithmic phase of growth. The *agr* two component systems consist of two transcripts, RNAII and RNAIII. The RNAII transcript

is under the control of the P2 promoter whereas RNAIII is controlled by another promoter, P3 [203] (Fig. 1.2.1.7). The RNAII transcript encodes four genes *agrA*, *agrC*, *agrD* and *agrB* [205] while the RNAIII encodes the delta hemolysin gene (Fig. 1.2.1.7) and also is believed to control most of virulence gene expression in *S. aureus* through an as yet unknown mechanism.

In the *agr* two component regulatory systems of *S. aureus* the bacteria produces the autoinducing peptide (AIP) when the bacterial density reaches a certain threshold. The autoinducing peptide is encoded by the *agrD* gene and the propeptide form processed by AgrB becomes the active autoinducing peptide. The AIP with its thiolactone functional domain is secreted after binding to AgrC, a transmembrane response-regulator domain. This binding leads to a phosphorylation cascade that transfers the phosphate from ATP to histidine and aspartate residues of AgrC and AgrA, respectively. Finally, this phosphorylation process results in the binding of AgrA to the target gene and induction of transcription processes of the cell [190, 206-212] (Fig. 1.2.1.7). AgrA was shown to bind DNA by recognizing a nucleotide consensus sequence of 5' ACAGTTAAG-3' separated by a 12 bp nucleotide sequence as spacer [213, 214]. RNAIII is mainly believed to be the effector of the *agr* regulon; however, the exact mechanism has yet to be defined [190, 215]. The RNAII product of the *agr* locus was shown to have sequence variability in the C-terminal of *AgrB*, the whole of *AgrD* and N-terminal of *AgrC* which results in four *agr* specificity groups [206, 216-218]. The AIP from one group inhibits the growth of another group member while it induces two component signal transduction pathways in the members of its own group [206]. It has been shown that the N-terminal of *AgrB* and the C-terminal of *AgrC* are much more conserved, whereas the intervening sequences were shown to be significantly variable [206, 217]. The variable sequences determine *agr* specificity. These *agr* group variations are expected to have differences in pathogenicity of *S. aureus* and each *agr* group might be relevant to a specific disease [217]. Some recent evidence showed that specific *agr* groups are known to cause certain specific diseases. For example menstrual toxic shock syndrome causing strain and necrotizing pneumonia causing strains belong to group III [206, 219], vancomycin intermediate resistant strain belongs to *agr* group II [220] and exfoliatin-producing strain belong to group IV [217, 221].

The accessory gene regulator (*agr*) system of *S. aureus* is induced by autoinducing peptide (AIP) produced by bacterial cells when their numbers reach certain level. Recent studies showed that RNAIII autoinducing peptide (RAP) and the target of RNAIII autoinducing peptide (TRAP) were also hypothesized to be involved in inducing *agr* two component signal-transduction pathways [222-224]; however, the detailed mechanism has yet to be shown experimentally.

In summary the various regulatory network generated at different phases of bacterial growth *in vitro* as well as *in vivo* allow the bacteria to adapt to their immediate environment very rapidly and specifically to achieve infection and/or successful survival in their host.

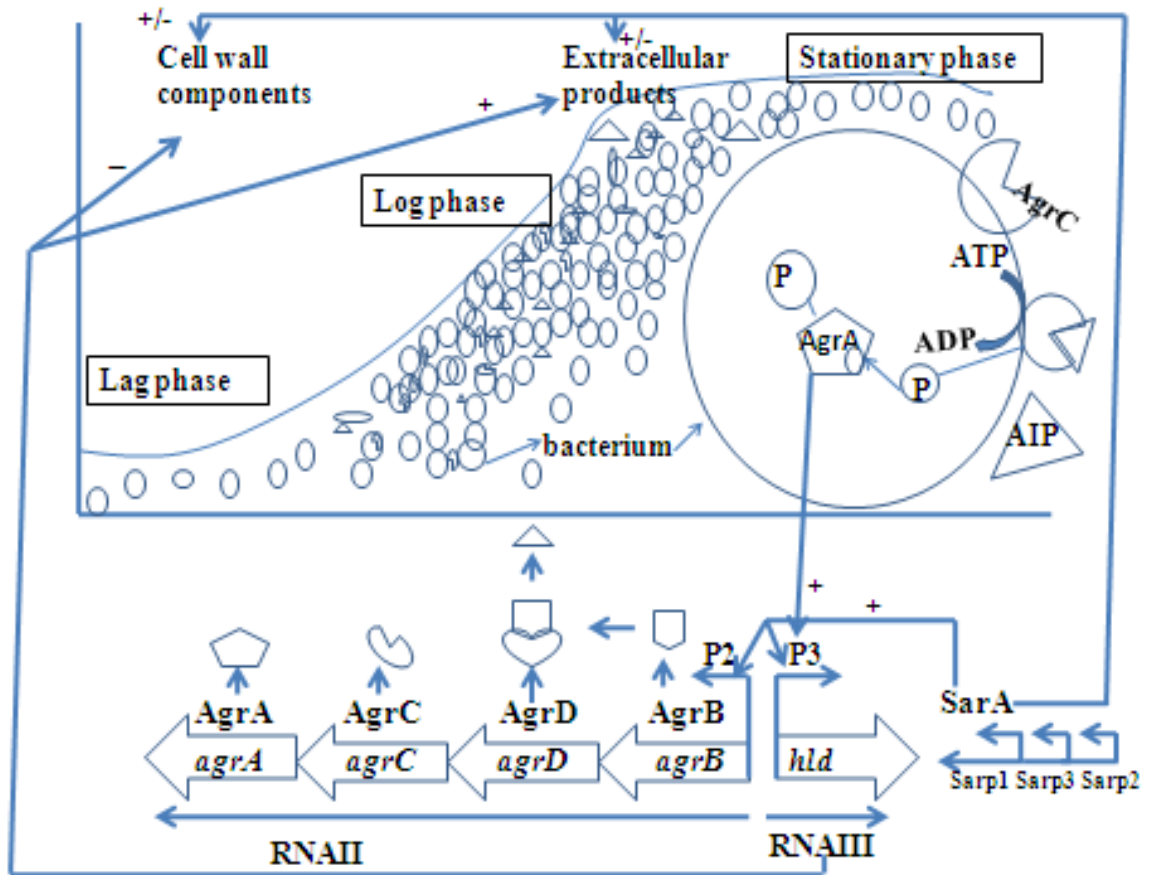


Figure 1.2.1.7. Growth phase dependent regulation of virulence gene expression in *S. aureus* by *sar* and the *agr* loci. The *agr* system comprises RNAII and RNAIII transcripts which are under the control of divergent promoters, P2 and P3. RNAII transcript consists of four genes, *agrA*, *agrC*, *agrD* and *agrB* which encodes AgrA, AgrC, AgrD and AgrB proteins, respectively. An RNAIII transcript consists of the *hld* gene that encodes delta hemolysin (Hld) and regulates P2-P3 promoters functions. The *agrD* encodes AIP which is the inducer of the *agr* two component signal transduction system at a certain level of bacterial cell density. AIP binds to AgrC, a transmembrane protein kinase which is a sensor of the two component signal. This binding leads to phosphorylation which transfers phosphate from ATP to histidine and aspartate residues of cytoplasmic domains of AgrC and AgrA, respectively. SarA also binds to P2-P3 promoters and regulates expression of several virulence genes in a bacterial growth phase dependent manner. SarA also directly regulates expression of some virulence factors at exponential and post exponential growth phases.



#### 1.2.1.8. Role of *agr* and *sar* in Biofilm Formation

Biofilms are a multilayered microbial community of cells that are attached to polymeric substances and manifest changes in bacterial metabolic reactions, physiology and gene expression [225]. A biofilm is formed through multiple steps which includes initial cell-to-cell attachment, adhesion and proliferation, maturation and removal from the layer [226]. Biofilm formation is an important factor in the pathogenesis of staphylococcal infections since it protects bacteria from phagocytic killing by polymorphonuclear phagocytes and antibiotics. In *S. aureus*, *poly-N-succinyl-β-1-6-glucosamine* (PIA/PNSG) and biofilm-associated protein (Bap) are involved in biofilm formation [173]. The PIA/PNSG is encoded by the *icaADBC* operon [173]. Biofilm formation is regulated through quorum-sensing in staphylococci and *agr* reduces biofilm formation and thereby decreases virulence of biofilm forming strains [227]. Moreover, synthetic RNAIII inhibiting peptides (RIP) were shown to down-regulate gene expression for biofilm formation and toxin production *in vitro* [228]. Moreover, *in vivo* prevention of virulence gene expression through *agr* resulted in decreased biofilm formation [229].

In another study an experimental model using indwelling medical device was developed to investigate the role of the *agr* system in biofilm associated infections by *S. epidermidis*. The *agr* mutant has been shown to colonize the device more than the isogenic wild type strain suggesting that *agr* inactivation increased biofilm formation [230]. These authors also explained that the Agr controls production of small peptides called phenol-soluble modulins (PSMS) responsible for bacterial detachment from biofilms and these modulins have been indicated to have strong proinflammatory properties. The exact role of SarA in biofilm formation is not clearly known, however, it has been reported that SarA-defective mutant MRSA and MSSA strains lost their biofilm formation capability [231]. The deletion of *agr* increased biofilm formation significantly in some MRSA and MSSA strains while its deletion had no effect in others [230-232]. O'Neill et al. [231] concluded that biofilm formation in MRSA strains is not affected by Ica; rather it involves a protein adhesin controlled by SarA and Agr. However, SarA-controlled PIA/PNAG plays a more important role in biofilm formation by MSSA strains. In another study biofilm forming cells was reported to express high levels of SarA which might

indicate positive regulation of biofilm formation by SarA [233]. Further detailed investigation is required to unravel these discrepancies.

### **1.3. Intramammary Defenses**

The mammary gland is protected by non-specific natural defenses as well as innate and acquired immune mechanisms. These are highly interactive and coordinated in order to provide optimal protection from mastitis.

#### **1.3.1. Natural Defenses**

The host is protected from pathogens gaining access to the intramammary area primarily by natural defense mechanisms. These natural defense mechanisms include closure of the teat canal by a group of smooth muscles called the rosette of Frustenburg, the keratin plug of the teat canal, and the antibacterial activity of esterified and non-esterified fatty acids which includes palmitoleic, myristic, and linoleic acids [234, 235]. Moreover, bactericidal cationic peptides are also indicated to have role in natural defense against intramammary infection. Increased patency of the sphincter during milk accumulation prior to parturition and removal of keratin have been correlated with increased susceptibility to bacterial colonization and invasion [235-237]. Colonization of the udder skin and teat opening and subsequent transfer into the intramammary area and establishment of infection can only be achieved when host natural defense systems are compromised.

#### **1.3.2. Intramammary Immunity**

##### **1.3.2.1. Innate Immunity**

Innate intramammary immunity comprises cellular and humoral components that are not specific to individual antigen molecules. The innate immune system recognizes the foreign antigen or pathogen associated molecular patterns not only by Toll-like receptors [238] but also by complement receptors, macrophage mannose binding lectin, ficolins and scavenger receptors

(Table 1.3.2.1). The innate immune system has three effector mechanisms, including the complement system, phagocytic cells, and antimicrobial peptides [239]. The cellular innate immunity comprises phagocytic cells (neutrophils and macrophages), natural killer cells and dendritic cells whereas humoral innate immunity includes the complement system,  $\beta$ -defensins, antimicrobial peptides, nitric oxide, lactoferrin, lysozyme, lactoperoxidase, and xanthine oxidase [240-242].

### **i. Cellular Innate Immunity**

Macrophages and T lymphocytes comprise a large cell population of the healthy mammary gland. Likewise, the number of neutrophils increases together with lymphocyte numbers in healthy udders as lactation progresses [243-245]. There is a direct correlation between high somatic cell counts and levels of protection against most mastitis causing agents [246-249]. In addition to mammary gland tissue has resident cells such as dendritic, natural killer and endothelial cells; however, their role in the protection of intramammary infection is not known.

The presence of invading pathogens can be detected early in the process of infection by innate immune receptors such as Toll-like receptors (TLRs) and others which are present on the surface of antigen presenting cells such as macrophages and dendritic cells. Toll-like receptors are of germline origin and they are evolved to recognize pathogen associated molecular patterns (PAMPs) which are conserved molecules that are specific to microorganisms [250]. The detection of invading microorganisms by Toll-like receptors results in the activation of defense genes and acquired immune responses [240, 241]. Currently, there are eleven TLR's known to exist in humans and mice which recognize different components of microbes (Table 1.3.2.1). Transcription of the genes coding for TLR2, and TLR4 was found to increase during mastitis in cattle [240, 241] and also infection with *S. aureus* increased expression of TLR4 [240, 251]. In addition to TLRs there are many other innate receptors on cell surfaces such as Dectin-1, CD14, FMLP (f-methionyl-leucyl-phenylalanyl) receptor, NOD1 (Nucleotide-binding oligomerization domain), and NOD2 as well as other humoral components such as LBP (LPS binding protein), CD14, collectins, properdin, C3b, C3bi, pentraxins [252].

Table 1.3.2.1. Receptors involved in innate immune recognition of pathogens

Receptor	Ligand	Reference
TLR1	Triacyl lipopeptides	[253]
TLR2	PG and LTA	[254, 255]
TLR3	Double stranded RNA of viruses	[256]
TLR4	LPS of Gram-negative bacteria	[257]
TLR5	Flagellin of bacteria	[258]
TLR6	LTA of Gram-positive bacteria	[255]
TLR7 and 8	Single stranded RNA viruses	[259, 260]
TLR9	CpG containing DNA	[261]
TLR10 and TLR11	Not well known	[262]
Collectins (MBL, MMR)	Binds to terminal mannose residues. Induces lectin-mediated complement activation pathway.	[250, 263-269]
SR-A family (MSR, MARCO)	Double-stranded RNA, LPS, and LTA. Bacterial cell wall and LPS	[250, 270, 271]
Pentraxins (SAP, CRP)	Binds to phosphocholine on bacterial surface and serve as opsonin, they also binds to C1q and induce the classical complement pathway.	[250, 272-274]
PKR	Double stranded RNA	[250, 275]
NOD proteins (NOD1 and NOD2)	Might bind to C-terminal leucine-rich repeat (LRR) of LPS	[250, 276, 277]
fMLP receptor	G-protein receptor on neutrophils, important in chemotaxis	[252, 278]
LBP and CD14	In their soluble form binds to LPS	[252]
C3b and C3bi	Binds to Mac-1/ICAM-1 on phagocytic cells	[252]

SAP = Serum amyloid protein, CRP = C-reactive protein, MBL = Mannan-binding lectin, MMR = Macrophage mannose receptor, Scavenger Receptor – A family, MSR = Macrophage scavenger receptor, NOD = Nucleotide-binding oligomerization domain, fMLP = f-methionyl-leucyl-phenylalanyl

Neutrophils are actively recruited to the site of infection and are the major cell type present in the mammary gland during early phases of *S. aureus* mastitis [136, 235]. After gaining access to the intramammary area *S. aureus* grows very rapidly and quick recruitment of

fresh blood neutrophils and opsonising antibody of IgG2 isotype is required [251]. These recruited neutrophils require bacteria opsonized by IgG2, IgM, or C3bi/C3b for efficient clearance of *S. aureus* infections [279-282]. Normal circulating neutrophils express CD62L (L-selectin) on their surface whereas activated neutrophils express E-selectin (CD62E) and P-selectin (CD62P) on vascular endothelial cells at the site of tissue infection [283, 284]. When neutrophils come in contact with the endothelium and detect proinflammatory cytokines, they become activated and increase expression of Mac-1 [251, 284-288]. This receptor allows neutrophils to bind to the endothelium and pass through to the site of injury. Neutrophil chemotaxis to the site of inflammation is influenced by several proinflammatory cytokines and chemo attractants such as IL-8, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, C5a and C3a [251, 289-292]. Neutrophil chemotaxis from blood into the mammary gland can take place within a few hours post infection [293-295]. The neutrophil recognizes opsonised bacteria through its Fc $\gamma$ R, CR1 and CR3 or Mac-1 receptors and phagocytose and degrade them by its pre-formed bactericidal components in its intracellular granules or by hydrogen peroxide and/or superoxide produced through a respiratory burst from glucose oxidation.

Macrophages are dominant cells in milk and tissues of healthy udders. Macrophages also phagocytose and kill bacteria using protease and reactive oxygen radicals [296, 297]; however, they are less in number during mastitis and also express Fc receptors on the cell surface [298]. It has been shown that macrophages secrete IL-12, which potentiates the development of IFN- $\gamma$  secreting cytotoxic CD8<sup>+</sup> cells [290].

## **ii. Humoral Innate Immunity**

Antimicrobial peptides are small polypeptides that can have an antimicrobial effect at normal physiological levels in the body [299-301]. They exist in several tissues including polymorphonuclear leukocytes, macrophages and mucosal epithelial cells. Major antimicrobial peptides known to exist in cattle include: defensins, cathelicidins and anionic peptides [302]. There are several cationic antimicrobial peptides and a few groups of anionic antimicrobial peptides in domestic animals [301]. Other mammalian antimicrobial peptides include histatins [303] and dermicidin [304]. Antimicrobial peptides have different mechanisms of killing

microbes. For example defensins induce ion channel formation [305], anionic peptides cause flocculation of intracellular contents [306] and batenecins affect transport and energy metabolism [307] (Table 1.3.2.2).

$\beta$ -defensins are antimicrobial peptides largely produced by polymorphonuclear cells [240, 308, 309]. *In vitro* stimulation of mammary epithelial cells with LPS and LTA induced high expression of  $\beta$ -defensin [310] and the transcription of  $\beta$ -defensin-5 increased during mastitis. In humans  $\beta$ -defensins form the main class of antimicrobial peptides that exist in epithelial cells [299]. Three  $\beta$ -defensins have been found in human skin including hBD-1, hBD-2 and hBD-3 [311-313]. Interleukin-1 from resident local monocytes induced the expression of  $\beta$ -defensin-2 in inflamed skin [314]. The hBD-1 is believed to be expressed constitutively [315]; however, some evidence showed increased expression during inflammation [316]. Despite increased expression of hBD-3 in inflamed skin and epithelia [311, 317], its regulation is not known; however, microbial molecules activated monocytes and lymphocytes stimulated the epidermal expression of all three human defensins [318]. The activation of hBD-3 was through transactivation of epidermal growth factor receptor [318]. Although antimicrobial peptides might have role in the control of intramammary infections they have not been evaluated for this activity.

Table 1.3.2.2. Bovine antimicrobial peptides

Antimicrobial peptide	Source	Function	Reference
<b>Cathelicidin</b>			
BMAP27	Bone marrow myeloid cells	Broad spectrum	[319-321]
BMAP28	“	“	[319-321]
BMAP34	Neutrophils, bone marrow myeloid cells, testis, spleen	“	[319,, 321, 322]
Bac5, Bac7	Neutrophils	Gram-negatives and positives	[319, 320, 323-326]
Indolicidin	Neutrophils	Gram-negatives and positives	[319, 327, 328]
Dodecapeptide	Neutrophils	Gram-positives and negatives	[319, 329, 330]
<b>Defensins</b>			
BNBD-1 – 3, 6 -11, 13	Neutrophils	Broad spectrum	[331-335]
BNBD4	Bone marrow, distal small intestine, trachea, colon, lung, spleen	“	[332, 334]
BNBD-5	Bovine alveolar macrophages	“	[334]
BNBD-12	Bone marrow, distal small intestine, trachea, colon	“	[332]
TAP	Nasal epithelium, trachea, bronchioles, alveolar macrophages	“	[336-338]
LAP	Alveolar macrophages, tongue	“	[334, 339]
EBD	Alveolar macrophages, intestine	“	[334, 340]
Anionic peptides	Lung	Gram-negative and positives.	[302, 306, 341]

Adapted from [301]

BNBD = bovine neutrophilic beta defensin, BMAP = bovine myeloid antimicrobial peptide, Bac = Bactenecin, TAP = Tracheal antimicrobial peptides, LAP = Lingual antimicrobial peptides. EBD = epithelial beta defensins.

Lactoferrin is an iron-binding bacteriostatic protein produced by epithelial cells and leukocytes. It chelates iron and makes it unavailable for microorganisms [235, 242, 342, 343]. It also releases lipopolysaccharides from Gram-negative bacteria by interacting with their cell membrane [344]. Some *in vitro* studies showed that *E. coli* and *S. aureus* are not affected by lactoferrin [345, 346].

Nitric oxide (NO) is a bactericidal agent produced by mammary epithelial cells and mononuclear phagocytic cells during mastitis [347-349]. Some reports also indicated that

enterotoxin-C of *S. aureus* increased NO production during mastitis [349] and a recent study reported that NO had no effect on intramammary defense [348]. The exact role of the NO during intramammary infection needs to be defined.

The complement system is a defense mechanism that generates effector molecules involved in both innate and acquired immunity through its classical, alternative and lectin activation pathways. They serve both as innate and acquired defense molecules. Their effector functions include lysis of bacteria, opsonization and the attraction of phagocytes to the site of complement activation [242, 350-352]. Complement is activated through three different pathways but all result in activation of C3 convertase which is a surface associated enzyme complex that cleaves C3. Cleavage of C3 activates deposition of C3b on the surface of microorganisms and release of C3a which is a potent chemoattractant for inflammatory cells. The C3b and its by product C3bi are deposited on the surface of microorganisms or antigens and allow them to be removed by phagocytic cells. Complement is reported to exist in the milk of healthy cows [351, 353] but the role of complement protection in the mammary gland is not clearly defined. The classical pathway is not functional in the mammary gland due to the absence of the C1q complement component.

Lysozyme (N-acetylmuramyl hydrolase) is a bactericidal protein that exists in milk and other mucosal secretions. It removes peptidoglycan from the cell resulting in bacterial death; however, its role in the intramammary protection is believed to be insignificant [242, 354].

Lactoperoxidase is a bacteriostatic agent for both Gram-negative and Gram-positive bacteria [352, 355]. Some reports indicated that bovine milk has about 30 µg peroxidase/ml [354]; however, its role in the intramammary defense is yet to be defined clearly. Xanthine oxidase was also reported as bacteriocidal agent in the milk but the specific role of this agent is not clearly known [242, 356].

#### **1.3.2.2. Acquired Immunity**

The main effectors of mammary gland immune defense against major mastitis-causing bacteria are blood derived neutrophils, opsonizing antibodies and effector T cells in cooperation



with cytokines [251, 357, 358]. Different cells in the mammary gland such as dendritic, natural killer,  $\gamma\delta$  T, endothelial, mammary epithelial, neutrophils, macrophages, CD4+ and CD8+ T cells and IgA producing plasma cells play an important role in local inflammatory responses [251].

### **i. Cellular Acquired Immunity**

Lymphocytes are able to detect antigens by their surface receptors, which determine their specificity, diversity, memory and self/nonself recognition [13, 235, 245]. They are divided into two main groups: T- and B-lymphocytes. The B-lymphocytes process and present antigen to T helper cells as well as produce immunoglobulins through stimulation by cytokines from T helper cells such as IL-2, IL-4, IL-6 and IL-10. These cytokines induce proliferation and differentiation of B-lymphocytes into either plasmocytes that produce antibodies or become memory cells. Their proportions among the total lymphocytes remain constant during mastitis [290].

The T lymphocytes include  $\alpha\beta$  CD4+ helper T lymphocytes,  $\alpha\beta$  CD8+ cytotoxic or suppressor T lymphocytes,  $\gamma\delta$  T lymphocytes and CD4+, FOXP3+, CD25+ regulatory T lymphocytes [359]. The dominant T lymphocytes in the mammary gland tissue and secretions of healthy human, porcine and bovine species are CD4+  $\alpha\beta$  T lymphocytes [360]. The peripheral blood and supramammary lymph nodes of healthy cows predominantly possess CD4+ lymphocytes [13, 361, 362]. During mastitis CD8+ T cells become the predominant cells recruited into milk. The functional importance for the increased number of CD8+ over CD4+ lymphocytes during mastitis is not defined. Based on the type of cytokines produced, the T-helper cell can stimulate either a cell-mediated (Th-1 type) or humoral (Th-2 type) immune responses [363]. Interleukin-2 (IL-2) and IFN- $\gamma$  are considered as the dominant cytokines during the Th-1 response whereas IL-4, IL-5 and IL-10 are characteristic of a Th-2 response. However, it is known that in cattle and humans as opposed to mice, IL-10 can be produced by Th1 and Th2 cells and regulate all their activities [364]. It is well understood that CD8+ cells can exert either cytotoxic or suppressor function [365]. As is the case for CD4+ cells, different cytokines were expressed by the two subtypes of CD8+ cells. IL-4 has been associated with the suppressor phenotype and IFN- $\gamma$  with the cytotoxic phenotype [365, 366].

Ruminants have increased numbers of  $\gamma\delta$  T-lymphocytes in the mammary gland compared to blood [367, 368]. However, their number decreases during the post partum period [369]. The role of these cells in the protection of intramammary infection is not known. Natural killer cells have cytotoxic activity and utilize their Fc receptors to achieve antibody-dependent cell-mediated cytotoxicity (ADCC). They express CD16 which is the Fc $\gamma$  receptor for IgG on their surface. Their killing activity is mediated through granule exocytosis, release of cytokines and receptor mediated antigen recognition [235, 370]. The role of NK cell in protection of intramammary infection is not known.

Regulatory T cells are either thymus derived lymphocytes or peripherally activated lymphocytes that regulate peripheral immune responses to self and non self antigens. The capability of the immune system to distinguish between self-antigens and non-self antigens is critical for the maintenance of immune homeostasis. The immune system has evolved a variety of mechanisms to achieve self tolerance. There are two major mechanism of tolerance; these are central tolerance and peripheral tolerance. Central tolerance is a clonal deletion of self-reactive T and B cells exposed to self antigens at the immature stages of their development [371-373] whereas peripheral tolerance is a mechanism by which self reactive T- lymphocytes that escaped clonal deletion at the central generative organ have been prevented from causing autoimmune diseases [374-376].

Recently, CD4<sup>+</sup> lymphocytes from mammary gland secretions of cows immunized with lectins and *S. aureus* antigen were found to be less responsive to these antigens compared to CD4<sup>+</sup> lymphocytes from peripheral blood of healthy cows [377]. This decreased response was believed to be due to inefficient antigen presentation by mammary gland antigen presenting cells and decreased efficiency of mammary lymphocytes. These authors also showed that concurrent with the decrease of CD4<sup>+</sup> cells there was an increase in the number of CD8<sup>+</sup> ACT2<sup>+</sup> cells from an antigen-challenged gland as compared to peripheral blood lymphocytes from healthy cows. They also showed that removal of CD8<sup>+</sup> ACT2<sup>+</sup> cells stimulated antigen responsiveness of lymphocytes from antigen challenged animals whereas stimulation of purified CD4<sup>+</sup> lymphocytes with different numbers of CD8<sup>+</sup> ACT2<sup>+</sup> lymphocytes decrease their responsiveness in a dose dependent manner. In another observation, cows with *S. aureus*

intramammary infection showed a change in the ratio of CD4+:CD8+ cells from 1.53 in peripheral blood to 0.85 in the mammary gland during *S. aureus* mastitis, which shows an increase in the number of CD8+ cells or decrease in the number of CD4+ cells or both during intramammary infection [245, 377, 378]. This CD8+ cell population was shown to have increased expression of the activation marker molecule, ACT2. After these early observations, these authors focused on *S. aureus* enterotoxin-C (SEC) due to the high association of SEC with mastitis isolates of *S. aureus* [111]. Staphylococcal enterotoxins, especially SEC, are suspected to be primary factors for the induction of CD8+ ACT2+ cells that down regulate the activity of CD4+ T cells [379]. Very recently, they showed that bovine peripheral blood mononuclear cells stimulated *in vitro* with SEC increased the number of CD8+ CD26+ cells (ACT3 is the bovine orthologue of CD26) and reversion of the CD4+:CD8+ ratio [380, 381]. In another study, bovine PBMC cultures stimulated *in vitro* with SEC to examine T cell apoptosis and proliferation status showed that SEC-stimulated bovine PBMC cultures had low nucleic acid synthesis during early stimulation but increased nucleic acid synthesis after 5 days of incubation compared to stimulation with ConA. On the other hand, nucleic acid synthesis in human PBMC cultures stimulated with SEC increased continuously. The bovine CD4+:CD8+ ratio in SEC-stimulated PBMC cultures showed increased CD8+ cells with decreased apoptosis compared to CD4+ T cells. These authors concluded that SEC induces a prolonged Th-2 biased response and this could explain the chronicity of *S. aureus* intramammary infection [382]. Recent evidence from an *in vitro* study on bovine peripheral blood mononuclear cells (PBMC) cultured with a low dose (5 ng/ml) of *S. aureus* SEC1 enterotoxin for 10 days [383] clearly showed stimulation of regulatory T cells which express typical surface molecules and express immunosuppressive cytokines. These authors showed that initially both CD4+ and CD8+ cells proliferate at equal rates but later on, CD8+ cells proliferate vigorously with increased expression of genes encoding CD25, CD152 (CTLA-4) surface molecules. They also showed an increase of mRNA of IL-10 and TGF- $\beta$  in SEC1-stimulated bovine PBMC cultures and they indicated that increased transcription of IL-10 and TGF- $\beta$  comes from the CD4+ CD25+ T cell subpopulations. FOXP3 mRNA also increased in this culture and this increase correlated with increased expression of CD152 and decreased expression of IL-2, which shows regulatory T cell involvement. They also showed that both SEC1 stimulated CD4+ and CD8+ cells suppress the proliferation of naïve PBMC exposed to heat killed *S. aureus* which is contrary to the previous observation which

indicated suppression of CD4+ cells by SEC-stimulated CD8+ CD26+ cells [382]. However Seo *et al.*, [383] showed that SEC-stimulated CD4+ cells induced suppression of naïve PBMCs through IL-10 and TGF- $\beta$  dependent mechanisms whereas CD8+ cells induced suppression did not involve IL-10 and TGF- $\beta$ . These *in vitro* findings add one more possibility for a mechanism of chronic intramammary infection by *S. aureus* if this can be proven under *in vivo* conditions.

Table 1.3.2.3. Regulatory T lymphocytes

Treg type	Origin	Phenotype	Cytokine expression	Mode of action	Reference
Natural Treg	Thymus	CD4+ CD25+ FOXP3+, CTLA-4 +	+/- IL-10 +/- TGF- $\beta$	Cell contact, membrane or soluble TGF- $\beta$ , secreted IL-10	[384-389]
Acquired Treg (Tr1 and Th3 cells)	Periphery	CD4+, FOXP3 intermediae expression, CILA-4 and CD25 (acquired with activation)	IL-10, IFN- $\gamma$ TGF- $\beta$ , IL-4	Secreted IL- 10, cell contact, membrane or soluble TGF- $\beta$	[390-392]

TGF= Transforming growth factor beta, Treg = Regulatory T cells, CTLA= cytotoxic T lymphocyte-associated antigen-4, Tr1 = T regulatory 1 cells.

## ii. Humoral Acquired Immunity

It is evident that both systemic and local humoral immune responses can contribute to mammary gland immune defences. The soluble effectors of the specific immune response are antibodies, cytokines and complement. In cattle, there are four classes of immunoglobulins known to influence the bacterial defense mechanism of the mammary gland. These are: IgG1, IgG2, IgA and IgM [393].

Immunoglobulin A (IgA) secreting B-cells are attracted to the mucosal surfaces after stimulation by the antigen at the mucosal associated lymphoid tissues. In humans and monogastric species, intramammary immunity is a part of the mucosal immune system so that immunization through any mucosal surface will induce immune responses in the mammary gland [394-398]; however, in ruminants it is not well defined whether intramammary immunity is part of mucosal immune system [399, 400]. Immunoglobulin A (IgA) in the milk is synthesized by B-cells, which are resident in the mammary gland. These lymphocytes migrate to the secretory epithelia after first interaction with their antigen in the mucosal associated lymphoid tissues. The initial recognition of antigen induces expression of specific surface receptors, addressins, also known as L-selectin and  $\alpha 4\beta 7$  integrin, which binds to homing receptors, MAdCAM-1, present on the vascular endothelial cells in secretory epithelia [401]. This event leads to an interaction between lymphocytes and endothelial cells which results in migration of mature IgA-secreting cells into the lamina propria below secretory epithelial cells [401, 402]. Covalently linked dimeric secretory IgA (sIgA) is transported across epithelial cells by the polymeric Ig receptor (PIgR) [343, 401]. Some studies reported a significant correlation between IgA concentration in milk and phagocytosis of *S. aureus* by bovine PMNs [251]. Other studies indicated that IgA does not act as an opsonin [403]. Nevertheless, all studies indicated that sIgA might have other functions such as prevention of the adherence of bacteria to the epithelium of mammary gland and/or neutralization of toxins produced by microbial pathogens.

Immunoglobulin-G (IgG) is produced at regional lymph nodes by IgG secreting B-cells, and secreted into blood and transferred from blood to the mammary gland across endothelial and epithelial layers. The exact mechanism of transportation from regional lymph nodes to the mammary gland is not known and some reports indicated that this transportation is achieved by a yet unknown prolactin-sensitive mechanism [343, 401]. It has been shown that prolactin inhibits IgG transfer into milk and also decreases the expression of an Ig-binding protein on mammary epithelial cells [343]. In bovine mastitis, IgG2 and IgM are known to be involved in opsonophagocytic clearance of *S. aureus* from the mammary gland [251]. The IgG1 is the dominant immunoglobulin in healthy milk and colostrum, especially during early lactation; however, its role as an opsonin for neutrophils is not proven [404]. Nevertheless, it might be involved in the prevention of bacterial colonization and neutralization of toxins [251]. Generally,

immunoglobulin-M (IgM) is the most effective opsonin for opsono-phagocytic removal of any foreign body or microorganisms but its role in the mammary gland is not clearly known.

Cytokines mediate, regulate and coordinate immune responses in the body through stimulation of activities of several cells which are either directly involved in immune effector function or produce immune effector molecules (or involve both functions). They form a network through which some humoral and/or cellular immune responses are regulated. Immune cytokines such as IL-2, IL-12 and IFN- $\gamma$  are produced by Th1 cells and they promote cell mediated immune responses whereas IL-4, IL-5 and IL-10 are produced by Th2 cells and are mediators of humoral immune responses. It has been shown that IL-12 is produced by macrophages and B-cells and it favors the Th1 type response [405] whereas IL-4 enhances the development of Th2 type responses [290, 406].

It is not clearly defined whether a Th1/Th2 bias exists during intramammary infection. However, a Th1 response can clear most intramammary infections. Similarly, a Th2 response might play role in preventing establishment of intramammary infections [251]. There are some reports which show cytokine profiles during *S. aureus* and/or *E. coli* mastitis. In an experimental infection of cows with *S. aureus* or *E. coli*, expression of proinflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  have been observed [407]. Analysis of milk and blood samples for cytokine expression in dairy cows immunized with *S. aureus* alpha toxin showed high expression of mRNA of IL-1 $\beta$ , IL6, TNF- $\alpha$ , IL-8 and IL-12 in all milk and blood samples [291]. These authors also showed that for milk-derived neutrophils, the expression of CD11b, and CD18 was increased which may indicate activation and chemotaxis of neutrophils. It has been shown that *in vitro* stimulation of mammary epithelial cells with lipopolysaccharide (LPS) from Gram-negative and lipoteichoic acid (LTA) from Gram-positive bacteria for 24 hs produced increased levels of mRNA expression for IL-1 $\beta$ , IL-8, TNF- $\alpha$ , CXCL6, and  $\beta$ -defensins in LPS-stimulated cells whereas no significant changes have been detected in LTA stimulated cells after 24 hs. However, LTA-treated cells have significantly increased expression of these transcripts after 4-6 hs of stimulation which remained elevated up to 24 hs [310]. It has been shown that during mastitis, proinflammatory cytokines such as IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  are produced and are involved in the regulation of inflammatory responses [290]. They also increase number

of blood and milk neutrophils as well as phagocytosis and bactericidal activity of neutrophils [235]. These cytokines regulate the inflammatory responses [408]. They induce vascular endothelial adhesion molecule expression locally on endothelial cells thereby enhancing neutrophil chemotaxis to the site of infection. Tumor necrosis factor alpha (TNF- $\alpha$ ) and IL-1 $\beta$  are potent inducers of fever and the acute-phase response. The acute phase reaction results in increased production of proteins such as LPS-binding protein (LBP), C-reactive protein, serum amyloid A, haptoglobin by hepatocytes [409]. These acute phase proteins might be involved in the defense mechanism of the mammary gland since they have host defense and antimicrobial activity and they can be used for early diagnosis of mastitis.

Interleukin-12 is associated with Th1 type responses by stimulating production of IFN- $\gamma$ , which in turn activates neutrophils and macrophages [410]. It increases the activity of mononuclear cells, especially lymphocytes including NK cells. Interferon gamma (IFN- $\gamma$ ) enhances the activity of Th1 cells and up-regulate production of IgG2 producing plasma cells. In the mammary gland experimentally infected with *S. aureus*, IL-12 increased 24 hs post infection [411, 412]. Interleukin-12 (IL-12) is an important cytokine for the differentiation of Th1 CD4+ cells [413]. It also enhances IgG2a production [413]. Moreover, significant mRNA transcripts of IL-12 have been observed during mid and late lactation, a time during which the mammary gland is relatively well protected. *In vitro* and *in vivo* studies indicated that recombinant bovine IL-2 may enhance the function of mononuclear cells within the mammary gland [235].

Colony stimulating factor (CSF) is produced by several cells such as fibroblasts, endothelial cells, macrophages and T cells and its function is to stimulate growth and differentiation of several hematopoietic cells [414]. Macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) regulate growth and differentiation of granulocytes and macrophages respectively. The roles of these growth factors, other than the normal growth initiation of respective cells, are not well known during mastitis.

Dairy cows are more susceptible to mastitis during the peri-parturient and early non-lactating periods. The increased susceptibility of dairy cows to mastitis during these periods can be due to several factors, some of which could be the influence of hormones (cortisol, which is

an anti-inflammatory hormone, estradiol, progesterone, etc.), dilution of immune effectors by milk, and the interaction between milk components and immune effectors which might decrease their activity [235, 251]. During the periparturient period, somatic cell counts increase at parturition and decrease at postpartum; however, the incidence of mastitis is very high during this period [235, 251]. During the postpartum period peripheral blood CD8<sup>+</sup> lymphocytes mainly produce IL-4 and they have less cytotoxic activity [136]. It has been shown that the CD4<sup>+</sup> enriched cultures of peripheral blood mononuclear cells isolated postpartum have increased IL-4 and IL-10 mRNA transcript expression [406]. This might indicate either a Th-2 biased response or immune suppression by regulatory T cells that leads to a high incidence of mastitis during these periods. In another study, mammary cell cultures isolated during the mid to late lactation period, during which incidence of mastitis is low, had increased IL-2 mRNA transcripts [406]. These authors also showed that depletion of CD4<sup>+</sup> lymphocytes decreased and enrichment of CD4<sup>+</sup> lymphocytes increased IFN- $\gamma$  transcripts in cultures of peripheral blood mononuclear cells isolated from mid to late lactation cows [406]. The existence of other cells that can secrete IL-4, such as CD8<sup>+</sup> suppressor cells, might justify the reason why CD4<sup>+</sup> depletion did not decrease IL-4 transcript levels in these cultures. Cells from mid-lactation dairy cows showed cytotoxic activity and they secreted high IFN- $\gamma$ , while CD8<sup>+</sup> lymphocytes from cows at postpartum showed no cytotoxic activity and secreted increased level of IL-4 [235, 240]. Studies on neutrophils gene activities [415] found that both immune response genes and the basic cellular genes for metabolism to generate energy were affected during the periparturient period [251].

Intramammary infections by most mastitis causing pathogens can be controlled by rapid recruitment of neutrophils from blood into the mammary gland after infection with a concomitant increase of IgG2 immunoglobulin in the gland for successful opsonophagocytic removal of the infecting microorganism. The protective intramammary immune responses for most mastitis causing pathogens are Th-1 dominated responses that express IFN- $\gamma$ , IgG2 and IL-2 with rapid recruitment of blood derived neutrophils and efficient opsonophagocytosis [251, 416-419]. Most mastitis vaccines induced Th-2 dominated immune responses that express IL-4, IL-10, high IgM and IgG1 with reduced Th-1 type inflammatory responses [251]. These Th-2 dominated responses did not clear invading intramammary pathogens. Increased numbers of stimulated Th-1 cells resulted in increased production of IFN- $\gamma$  that activate neutrophils and



macrophages to increase their surface Fc receptors and phagocytosis induced respiratory burst activity for antibody-dependent and antibody-independent killing of invading pathogens [251, 416-419]. However, massive infiltration of neutrophils also causes severe damage to the mammary gland tissue; therefore, a balanced response that protects against infection with minimal damage to the mammary gland tissue is required.

#### **1.4. Treatment, Control and Prevention of Bovine *Staphylococcus aureus* Mastitis**

##### **1.4.1. Treatment of Bovine *Staphylococcus aureus* Mastitis**

Mastitis due to *S. aureus* can be prevented or reduced in incidence. Antibiotic therapy can be applied to subclinical or clinical cases of mastitis during lactation or during the dry period. However, different treatment regimes failed to cure *S. aureus* intramammary infection [47, 57, 420, 421]. Antibiotic treatment is ineffective since only 20-70% of the dry period therapy is effective and only 10-30% of *S. aureus*-infected quarters are cured during lactation [422]. *S. aureus* adhesion and invasion into mammary epithelial cells allows the bacteria to escape the effect of immunity and antibiotics by being hidden in the intracellular niche and thereby causing chronic recurrent intramammary infections. Moreover, the appearance of electron transport-defective, drug-resistant small-colony variants which can stay in intracellular areas makes the control and prevention of *S. aureus* mastitis more difficult. It has been reported that in a total of 58 studies that examined results of treatment of *S. aureus* mastitis the cure rates were 14-100% for dry cow therapy with an average of 63% and 26-92% for clinical mastitis with an average of 54% [423]. These authors also reported that out of 16 trials on subclinical *S. aureus* mastitis the cure rate was 17- 95% with an average of 48%. Although dry period therapy seems better as compared to lactation therapy the overall success rate of mastitis treatment is not promising indicating the need for development of better prophylactic and therapeutic strategies.

##### **1.4.2. Control and Prevention of Bovine *Staphylococcus aureus* Mastitis**

Improved control of bovine *S. aureus* mastitis can be achieved by combined application of dry-cow therapy and milking-time preventive hygienic techniques. Standard hygienic milking

protocols and dry cow mastitis therapies have been introduced for the control and prevention of intramammary infection [424]. Good milking practices include: wash udder and use one service towel per cow to dry the udder, pre- and post-milking teat dipping into disinfectant solutions, use of gloves by milkers, and milking unit disinfection. The basic mastitis control program is based on five-point mastitis control plans, which include: milking practice with regular testing and maintenance of the milking machine, application of a teat disinfectant immediately after removal of the milking machine, routine antibiotic therapy for all cows after each lactation, treatment and documentation of all quarters with clinical mastitis, and culling of cows with chronic or recurrent mastitis [425]

The goals of this five-point control plan are targeted at reducing exposure, duration and transmission of intramammary infection by bacteria. This has markedly reduced the incidence of contagious mastitis but has had little effect on the incidence of environmental mastitis [426]. This may be due to inadequate knowledge of the pathogenesis of the infection, insufficient clearance of the infection during treatment or inadequate awareness of the importance of different strains of *S. aureus* as well as vectors and mechanism of spread. It also may relate to the manifestation of certain strains of highly transmissible *S. aureus* [38]. Some studies suggest that there are strains that are common to several herds, even in separate states [427, 428] or countries [429].

The isolation and culling of infected cows is also an effective control procedure for *S. aureus* mastitis [423]. However, these techniques are time-consuming, labour intensive and expensive. Therefore, a better strategy is needed to reduce the prevalence of *S. aureus* mastitis in dairy herds.

#### **1.4.2.1. Vaccination**

Despite several successful vaccine trials conducted with different commercialized [430, 431] and non-commercialized vaccines [93, 432] (Table 1.4.2.1) all field trials with these vaccines have been unsuccessful [6]. These include vaccines developed from live attenuated or

killed *S. aureus* cells, toxins ( $\alpha$  and  $\beta$  hemolysins), capsular polysaccharide, protein A and fibronectin-binding proteins [433-438]. Vaccination with autogenous whole cell bacterins and specific antigen fractions do not elicit heterologous protection [6, 93, 109, 439-442]. However, vaccines containing combined antigenic factors [170] from *S. aureus* surface components have shown promise if the antigen, adjuvant and route of delivery are appropriate. Major problems affecting the successful development of protective *S. aureus* vaccines against bovine *S. aureus* mastitis are strain variation, the presence of capsular polysaccharide in most pathogenic strains which does not allow recognition of antibody coated *S. aureus* by phagocytic cells, dilution of immune effectors by milk [6, 443], interaction between milk components and immune effectors [444] that reduce their effectiveness, and the ability of *S. aureus* to attach and internalize into mammary epithelial cells. There is an increasing need for the development of better vaccines that overcome these problems. A better understanding of the natural and acquired immunological defenses of mammary gland coupled with detailed knowledge of the *S. aureus* pathogenesis should lead to the development of improved methods of reducing the incidence of mastitis in dairy cows.

Table 1.4.2.1. *Staphylococcus aureus* vaccines tested

<b><i>S. aureus</i> vaccine</b>	<b>Type of vaccine</b>	<b>Immunogenic effects</b>	<b>Reference</b>
Whole <i>S. aureus</i> cell lysate encapsulated in biodegradable microspheres	Whole cell lysate	Induced antibodies that were more opsonic for neutrophils and inhibited adhesion to mammary epithelium.	[93]
Whole cell lysate from two strains ( $\alpha$ and $\alpha + \beta$ hemolytic) plus supernatants from non-hemolytic strain	Whole cell lysate from two strains	Vaccinated cows had 70% protection from infection compared to less than 10% protection in control cows	[439]
MASTIVAC I	Whole cell lysate	Improved udder health in addition to specific protection against <i>S. aureus</i> infection	[431]
Live pathogenic <i>S. aureus</i> through IM route	Live pathogenic <i>S. aureus</i>	Induce activation of immune cells in mammary gland and blood.	[109]
Fibronectin binding protein and clumping factor A	DNA primed and protein boosted	Induced cellular and humoral immune responses that provide partial protection against <i>S. aureus</i>	[445]
Protein A of <i>S. aureus</i> with green fluorescent protein	DNA	Induced humoral and cellular immune responses	[446]
Plasmid encoding bacterial antigen $\beta$ -gal	DNA	Induced humoral and cellular immune responses	[447]
Polyvalent <i>S. aureus</i> bacterin	Protein	Eliminated some cases of chronic intramammary <i>S. aureus</i> infections	[448]
Five-isolate-based <i>S. aureus</i> bacterin (Lysigin), three-isolate experimental bacterin and five-isolate experimental bacterin	Bacterin	Lysigin reduced the clinical severity and duration of clinical disease. None of experimental bacterins has significant effect.	[430]
Polyvalent <i>S. aureus</i> bacterin combined with antibiotic therapy.	Bacterin	<i>S. aureus</i> intramammary infection cure rate increased	[449]
Whole cell trivalent vaccine containing CP types 5, 8 and 336 with adjuvants (FICA or Alum	Whole cell lysate	Elicited antibody responses specific to the 3 capsular polysaccharides (increased IgG1 and IgG2 in all vaccinated animals	[432]
CP conjugated to a protein and incorporated in poly microspheres and emulsified in Freund's incomplete adjuvants	CP types 5, 8 and 336	Cows in both groups produced increased concentrations of IgG1, IgG2 antibodies, neither groups produced an increase in IgM. Sera from cows immunized with conjugates in microspheres induced sustained increased phagocytosis. Immune sera from both groups decreased bacterial adherence to bovine mammary epithelial cells.	[170]
Polysaccharide-protein conjugates emulsified in Freund's incomplete adjuvant.	Polysaccharide-protein conjugate		

#### 1.4.2.2. DNA Vaccines.

Most vaccination strategies for prevention against mastitis have focused on the enhancement of humoral immunity. The developments of vaccines that induce a protective Th1 type cellular immune response in the mammary gland have not been well investigated. The ability to induce cellular immunity, especially neutrophil activation and recruitment into the mammary gland is one of the key strategies in the control of *S. aureus* mastitis. One such strategy is use of DNA vaccines that contain pathogen-specific genes with known antigen-presentation ability for quick activation of Th1 immune responses and that also induce increased production of opsonizing IgG2 antibody by B cells. It has been shown that genes in a plasmid vector can be injected into animals with the subsequent expression of the encoded protein [450] and that an immune response could be induced to the proteins [451-454]. In another study intradermal injection of DNA encoding a green fluorescent protein (GFP) gene resulted in the production of antibody to GFP [455]. In addition it has been shown that DNA-based vulvo-mucosal immunization in cattle stimulated both cellular and humoral immune responses [456, 457]. These authors also showed that the circulating antigen presenting cells of the dermis migrated to the injection site, indicating their involvement in antigen presentation [457].

DNA vaccines have many advantages over other conventional vaccines. They are easy and economical to produce, have no requirement for an adjuvant [458] and thus, no injection site reaction problems due to adjuvant occur [459], induce balanced Th1/Th2 like immune responses which is important for the control of several viral infections [460, 461], can induce immune responses in neonates without interference by maternal antibody [462, 463], and can be given *in-utero* [464]. Despite all these advantages, DNA vaccines are less efficient in humans and large animals and induce weaker humoral responses. The reason for this low efficiency is believed to be due to poor transfection of host cells by DNA. The improvement of DNA delivery with appropriate vector selection is expected to improve the efficacy of DNA vaccines in large animals. It has been hypothesized that immune responses to DNA vaccines in large animals can be improved by improving three major components which are a promoter of the gene, gene of interest for immune induction and back bone of the plasmid for delivery of construct into cells [461]. These authors also proposed a model system where an improved promoter with

appropriately enhanced antigen expression system combined with an optimal delivery route, will induce balanced Th1/Th2 responses in large animals and humans. For example, as one means to optimize DNA immune response in large animals, it has been successfully shown that gene gun or suppository delivery of bovine herpes virus -1 glycoprotein-D (BHV-1 gD) to the vaginal mucosa induced protective immune responses both in the vaginal and nasal mucosa, indicating that vaccination at vaginal mucosa can induce protection at other common mucosal surfaces [461]. These authors also showed that simultaneous injection of two plasmids encoding different antigens did not result in significant interference. They suggested that various combinations of antigen delivery protocols could increase immune response to DNA vaccines in large animals. In another study DNA vaccination with an improved bicistronic plasmid encoding a fusion of two sequences from *fnbP* and *clfA* genes separated by an Internal Ribosomal Entry Site (IRES), followed by a booster vaccination with respective proteins, resulted in both cellular and humoral immune responses that gave partial protection of mammary gland from staphylococcal mastitis [445].

## **2. HYPOTHESIS, OVERALL GOALS AND SPECIFIC OBJECTIVES**

### **2.1. Hypothesis: Strengthening Local Intramammary Immunity Will Protect against Bovine *S. aureus* Mastitis**

Our hypothesis is based on the fact that most of the previous *S. aureus* mastitis vaccinations were focused on inducing protective systemic humoral immune responses rather than enhancing local immune responses in the mammary gland. Vaccination strategies that can also enhance cell-mediated immunity in the mammary gland have not been pursued. Moreover, most *S. aureus* vaccines were composed of killed bacterial cells or bacterial products that elicit protection against a limited number of strains. It is evident that both systemic and local humoral immune responses can contribute to the mammary gland immune defenses. However, we believe that strengthening local immune responses using highly immunogenic molecules conserved among pathogenic strains of *S. aureus* combined with the best adjuvant and appropriate selection of the route of administration that induce maximum stimulation of the immune system will protect against bovine *S. aureus* mastitis.

### **2.2. Overall Goals and Rationale of this Study**

The complex interaction between the intramammary pathogens and mammary gland defense mechanisms are not well understood. Moreover, the protective host immune responses needed to eliminate pathogens from the mammary gland and how to enhance protective intramammary immunity before mastitis is established are not well defined. Therefore, the overall goals of this study were:

- A. To improve our understanding of intramammary immunity using GapB and GapC proteins of *S. aureus* as model antigens
  
- B. To determine the regulation of expression of the *gapB* and *gapC* genes and their role in virulence.

### 2.3. Specific Objectives

1. To test immune response to GapB, GapC and GapC/B proteins in mice.
2. To test immune responses in mice after vaccination with plasmids, *gapB*, *gapC* and *gapC/B* encoding GapB, GapC and GapC/B proteins respectively.
3. To optimize the immune response in the mammary gland of cows
4. Monitor duration of immune responses in cows with the best immunogenic proteins and adjuvants selected from the above mentioned mice experiments (GapC/B and VSA3).
5. To determine the role of GapC in the virulence of *S. aureus*.
6. To determine the role of environmental conditions and global virulence regulators (*agr/sar* loci) in the regulation of expression of the *gapC* and *gapB* genes in *S. aureus*



### 3. MATERIALS AND METHODS

#### 3.1. Bacterial Strains, Culture Media, Growth Conditions and Preparation of Competent Cells

##### 3.1.1. Bacterial Strains and Growth Conditions

The *S. aureus* strains SA10 and SA12 were isolated from clinical bovine mastitis cases in dairy farms in Saskatoon, SK, Canada [75]. *S. aureus* strain 16 (SA16) was also from a bovine clinical mastitis case and it was obtained from Dr. Lorraine M. Sordillo Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, MI, USA). The *S. aureus* strains RN6390 and its isogenic *gapC* mutant strain H330 (RN6390 *gapC::tet* ) [79] were obtained from Dr. David Heinrichs (Microbiology & Immunology, University of Western Ontario, London, Ontario, Canada), The *S. aureus* strain ALC132 (RN6390) and its isogenic mutants *agrA*, ALC133 (RN6390 *agrA::erm*), *sarA*, ALC136 (RN6390 *sarA::erm*), *agr/sar*, ALC135 (RN6390 *agr/sar::tet/erm*) were obtained from Dr. Ambrose L. Cheung (Department of Microbiology, Dartmouth Medical School, Hanover, NH, USA). The identities of the strains were confirmed at VIDO using the API Staph kit (bioMérieux, Marcy-l'Étoile, France). The *S. aureus* strains were grown in tryptic soy broth or agar (DIFCO, Becton-Dickinson, Sparks, MD, USA), tris minimal succinate (TMS) broth (defined growth media containing succinate as carbon source) [465], tris minimal glucose (TMG) broth (defined media with glucose as carbon source) and whey. Whey was prepared from whole milk as described in section 3.1.2 and filter-sterilized. Tris minimal succinate medium contains: 4% (v/v) of a sterile tris salt stock (NaCl 145 g/L), KCl (92.5 g/L), NH<sub>4</sub>Cl (27.5 g/L), Na<sub>2</sub>SO<sub>4</sub> (3.55 g/L), KH<sub>2</sub>PO<sub>4</sub> (6.8 g/L), tris base (12.1 g/L), sodium succinate (16.6 g/L), adjusted to pH 7.4 and supplemented with filter sterilized tryptophan (0.02 g/L), cysteine (0.022 g/L), thiamine (0.0169 g/L), nicotinic acid (0.00123 g/L), pantothenic acid (0.0005 g/L), biotin (0.00001 g/L), MgCl<sub>2</sub> (0.0953 g/L) and CaCl<sub>2</sub> (0.0111 g/L). Tris minimal glucose media has the same components as TMS and prepared exactly as for TMS except for the replacement of succinate with glucose (5 g/L). *S. aureus* strains were grown in tryptic soy broth and whey at pH 5 and pH 7.4 when necessary. Tryptic soy broth at pH 7.4 was prepared with 0.1 M of 3-(N-morpholino) propanesulfonic acid (MOPS) and continuously buffered with MOPS during bacterial growth. To maintain anaerobic and microaerophilic

conditions cultures were grown in an aerobic jars containing BBL™ Gaspack™ with anaerobic and microaerophilic system envelopes respectively (BD Company, Sparks, MD, USA). The *gapC* mutant strain H330 (RN6390 *gapC::tet*) [79] was grown in TMS broth or agar at 37 °C in the presence of antibiotics (tetracycline 4µg/ml), when necessary. The TMS solid medium was obtained by the addition of Bacto agar (DIFCO, 15 g/L). *Escherichia coli* strains *E. coli* BL21 (DE3) (Novagen Inc. Madison, WI, USA), SG13009 (pREP4) [466] and DH5-α were grown in Luria-Bertani broth or agar (DIFCO) at 25 °C or 37 °C, in the presence of antibiotics (100 µg of ampicillin per ml or 50 µg/ml of carbencillin or 50 µg/ml of kanamycin per ml) when necessary.

Table 3.1.1.1. Bacterial strains and plasmids used in this study

Name	Description	Reference
<b><i>S. aureus</i> strains</b>		
SA10 , SA12	Bovine mastitis clinical isolates	[75]
SA16	Bovine mastitis clinical isolates	VIDO
RN4220	Restriction enzyme deficient 8325-4 mutant	[467]
RN6390	Prophage cured wild type strain, human isolate	[205]
H330	RN6390 <i>gap:: tet; Tc<sup>r</sup></i>	[79]
ALC133	RN6390 <i>agrA:: erm; Em<sup>r</sup></i>	[468]
ALC136	RN6390 <i>sarA:: erm; Em<sup>r</sup></i>	
ALC135	RN6390 <i>sar/agr:: tet/Emr, Tc<sup>r</sup>,Em<sup>r</sup></i>	
<b><i>E. coli</i> strains</b>		
DH5-α	Host for plasmid	Laboratory stock
BL21(DE3)	Used to express the histidine-tagged Gap protein genes.	Laboratory stock
SG13009 (pREP4)	<i>F<sup>+</sup> his pyrD Δlon-100 rpsL</i>	[466]
<b>Plasmids</b>		
pBISIA-24	Eukaryotic cell expression vector	[469]
pQE-30	Prokaryotic cell expression vector	Qiagen

### 3.1.2. Preparation of Whey from Milk

Whey was prepared as follows: Milk from 4 quarters of the cow was pooled in equal amounts and 1 rennet tablet, purchased from a health food store (Mom's nutrition, Saskatoon,

Canada), was homogenized in 40 ml of water or ¼ tablets in 10 ml. A 100 µl aliquot of the rennet mix was added to 2 ml of milk or 500 µl to 10 ml of milk. Samples were stirred with a wooden applicator stick. The tip of the stick was broken off so that the lid would fit back on the tube and the tubes were incubated for 2-6 hs (usually 4 hs) at 37 °C until a milk clot formed. After the milk clot formed, the tubes were centrifuged at 3000 X g for 20 min and the milk formed three layers as follows: fat, whey and curd at the top, middle and bottom of the tube respectively. The whey was collected carefully using a Pasteur pipette after removing the top fat layer.

### **3.1.3. Growth Conditions of *Staphylococcus aureus* Strains RN6390 and H330 for *in vitro* Adhesion and Invasion Assays and *in vivo* Challenge Trials.**

A 50 ml aliquot of tryptic soy broth (TSB) and tris minimal succinate (TMS) broth was inoculated with an overnight culture of 0.5 ml of *S. aureus* strains RN6390 and H330 respectively, in 125 ml flasks and incubated at 37 °C with shaking. The optical density (OD) of the cultures was taken at a wavelength of 600 nm using a spectrophotometer (Fisher Scientific Company, Ottawa, Ontario, Canada) and growth curves of OD<sub>600</sub> versus time were constructed. When cells were at the exponential phase (OD<sub>600</sub> of 0.4-0.5, usually within 2:30-3:30 hs) the growth was stopped by placing the flask on ice. Cells were collected by centrifugation at 3000 X g for 10 min at 4 °C. The cell pellet was suspended in 45 ml cold sterile PBS and centrifuged at 3000 X g for 10 min at 4 °C. Finally, the cell pellet was suspended in 5 ml cold sterile PBS, counted and diluted to 2 X 10<sup>7</sup> CFU/ml in MAC-T media {Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (DIFCO), 5µg/ml insulin, and 1µg/ml hydrocortisone (Sigma, St. Louis, MO, USA)} without gentamicin, and kept on ice until infused into the mammary gland. The serial dilutions of the bacterial suspensions were plated on TSA and TMS agar plates for RN6390 and H330 respectively to determine viable counts.

### **3.1.4. Infection**

To determine the number of attached and internalized RN6390 and H330, into MAC-T cells, the bacterial suspensions were used to inoculate MAC-T cell cultures at a multiplicity of infection (M.O.I) of around 20 to 50 CFU of *S. aureus* strains RN6390 or H330 per 1 MAC-T cell as follows: The spent media was removed from the 6 wells plate containing MAC-T cells

and a 2 ml aliquot of fresh MAC-T culture media was added to each well. Finally, 0.5 ml of the *S. aureus* suspension were added to each well and incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere for 1 h.

### **3.1.5. Determination of the Number of Total RN6390 and H330 Attached and Internalized into MAC-T Cells**

After MAC-T cells were co-incubated with *S. aureus* strains RN6390 or H330 for 1 h, aliquots of the culture supernatant were removed, serially diluted and plated for viable counts. The wells were washed 3 times with 2.5 ml of cold PBSA (PBSA = 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na<sub>2</sub>PO<sub>4</sub>, 0.2 g/L KPO<sub>4</sub> to 1000 ml distilled water) each time. Finally, 1ml of a solution containing 0.5% saponin and 0.025% trypsin (Sigma) was added to each well and plates were incubated at 37 °C for 10 minutes to lyse MAC-T cells. The lysate was serially diluted and plated for viable counts.

### **3.1.6. Determination of the Number of Invasive RN6390 and H330**

After 1 h of incubation of RN6390 or H330 with MAC- T cells, aliquots of the culture supernatants were removed, serially diluted and plated for viable counts. Each well was washed 3 times with pre-warmed MAC-T media with gentamicin (50 µg/ml). To kill extracellular bacteria 2.5 ml of media containing gentamicin (200 µg/ml) (Sigma) and penicillin (20 µg/ml) (Invitrogen Canada Inc., Burlington, Ont, Canada) was added to each well and incubated at 37 °C for 1 h. To monitor bacterial killing aliquots of the supernatants were taken, serially diluted and plated for viable counts. To determine the number of intracellular bacteria each well was washed 3 times with 2.5 ml of cold PBSA and MAC- T cells were lysed by treating with 1 ml of a solution containing 0.025% trypsin and 0.5% saponin in PBS and incubated for 10 minutes at 37 °C. Serial dilutions were made of the suspension and plated for viable counts.

### **3.1.7. Preparation of *Staphylococcus aureus* Cells for Electroporation and Electroporation Protocol**

An overnight culture of *S. aureus* strain RN4220 was diluted 100 fold in 500 ml TSB and incubated with shaking at 37°C until the culture reached an OD<sub>600</sub> of 0.4-0.5 (exponential phase). *S. aureus* cells were washed 3 times with ice cold 500 mM sucrose and suspended in a

final volume of 2 ml 500 mM sucrose. The *S. aureus* cell suspensions were aliquoted into pre-chilled microfuge tubes (60 µl each) and frozen in a liquid nitrogen or dry ice/ethanol bath. *Staphylococcus aureus* cells suspensions were electroporated in a 0.2 cm gap cuvette (Bio-Rad, Life Science Research, Mississauga, Ont, Canada) at a voltage of 2.5 Kv, capacitance of 25 µF and resistance of 100 Ω. A 50 ng of DNA was used for electroporation of *S. aureus*. The bacteria were recovered in non-selective medium, in this case TSB for 4 hs. Erythromycin was added to a 0.05 µg/ml and cells were allowed to recover for an additional 2 hs. Finally, aliquots of the suspension were plated on TSA with 3 µg/ml erythromycin.

### **3.1.8. Preparation of *Escherichia coli* for Electroporation and Eletroporation Protocols**

All *E. coli* transformation experiments involved electroporation. To prepare *E. coli* cells for electroporation an overnight culture in Luria Bertani broth (LBB) was diluted 1:100 in 500 ml of fresh broth and grown to early logarithmic stage ( $OD_{600} = \sim 0.2$ ). *E. coli* cells were collected by centrifugation at 4424 X g for 15 minutes at 4 °C. The cells were washed 3 times in 500 ml ice-cold sterile deionized water, suspended in 10% cold glycerol and stored in 50 µl aliquots at -70 °C until use. Bacterial cells were transformed by electroporation using a 1 mm gap cuvette (Bio-Rad) at 1.8 kV, 25 µF, 200 Ω by a gene pulser (Bio-Rad) using 50 µl of bacterial cells prepared as described above. A 50 ng of DNA was used for transformation of *E. coli*.

### **3.2. Culturing of Bovine Mammary Epithelial Cells**

Transformed mammary epithelial cells (MAC- T ) [470] were cultured as described [471] in 75 cm<sup>2</sup> tissue culture flasks (Corning Inc. Corning, NY, USA) to confluent monolayers. Cells were treated with 0.5% (w/v) trypsin (GIBCO BRL) in versene buffer (0.2 g/L sodium-EDTA, 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na<sub>2</sub>HPO<sub>4</sub>, 0.2 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.2 g/L glucose), resuspended in fresh DMEM containing 10% (v/v) of fetal bovine serum (FBS) (GIBCO BRL) and diluted to 5 X 10<sup>5</sup> cells/ml with DMEM. For adhesion and invasion experiments, 10<sup>6</sup> cells were added to each well of 6-well tissue culture plates and incubated at 37 °C in a 5% CO<sub>2</sub> (v/v) atmosphere for 18 hs.

### **3.3. DNA Preparation, Plasmid Construction and Manipulation**

#### **3.3.1. Genomic DNA, PCR and Plasmid Purification Protocols**

Genomic DNA from strains of *S. aureus* was purified with a Nucleospin Tissue Kit (Clontech, Palo Alto, CA, USA) with the addition of 5U/ml lysostaphin (SIGMA, St. Louis MO, USA.) and used as PCR template. PCR reactions were carried out using specific primers for *gapC* and *gapB* (Table 3.3.1). The PCR conditions were as follows: initial denaturation step at 95 °C for 4 minutes; followed by 40 cycles of 95 °C 1 minute, 50 °C 1 minute, 72 °C 1 minute each, and finished by incubation at 4 °C for 1 h. Aliquots of the PCR reactions were loaded on a 1% agarose gel, separated by electrophoresis, and stained with ethidium bromide. The PCR fragments were purified with the QiaQuick PCR purification kit (Qiagen, Mississauga, ON, Canada) and analyzed on a 1% agarose gel. Plasmid DNA was extracted using the Qiagen plasmid purification kit (Qiagen) following the manufacturer's instructions and analysed by restriction endonucleases as well as by sequencing of the inserted DNA

#### **3.3.2. Construction of Plasmids Encoding GapC, GapB and GapC/B Used for DNA Immunizations.**

The plasmids used in DNA immunizations were constructed using a derivative of pBISIA-24 [469]. This plasmid has regulatory sequences for expression of genes in eukaryotic cells, including the HCMV-IE1 promoter, intron A and the polyadenylation signals from the bovine growth hormone gene. The plasmid also contains CpG ODN motifs which have previously been shown to enhance the quality and quantity of the immune response following vaccination with plasmid DNA [469]. The *S. aureus gapB* gene, contained in a 1032 bp fragment obtained by PCR using the primers Sa7a and Sa8a (Table 3.3.1), was ligated to the 4393 bp *EcoRI/XhoI* fragment of pBISIA-24. The resultant plasmid was named pBISIA-*gapB*. The *S. aureus gapC* gene, contained in a 1025 bp *SmaI/XhoI* fragment obtained by PCR using the primers Sa5 and Sa10 (Table 3.3.1) was ligated to the 4406 bp *SmaI/XhoI* fragment of pBISIA-24. The resultant plasmid was named pBISIA-*gapC*. The 1938 bp *BamHI/SalI* fragment of pQE-SAgapCB2 encoding the *S. aureus gapC/B* chimeric gene [472] was ligated to

the 4357 bp *Bam*HI/*Xho*I fragment of pBISIA-24 and the resultant plasmid was named pBISIA-*gapC/B*.

### **3.3.3. Construction of Plasmids Encoding GapB, GapC and Chimeric GapC/B Recombinant Proteins for Expression and Purification in *Escherichia coli*.**

The PCR pair Sa-1 and Sa-2 (Table 3.3.1.) was used to amplify a *ca.* 1.1 kb DNA fragment from SA10 encoding the *gapC* gene and this fragment was digested with *Bam*HI and *Kpn*I. The resultant 1048 bp fragment was cloned into pQE-30 (Qiagen), an *E. coli* expression vector that adds a 6 x histidine tag at the NH<sub>2</sub> terminus of the GapC protein for purification by affinity chromatography. The resultant plasmid was named pQE-SA*gapC*. The primer pair Sa-3a and Sa-4 (Table 3.3.1) was used to amplify a *ca* 1 kb DNA fragment of SA10 encoding the *gapB* gene without the first 39 bp of the coding region. This fragment was digested with *Kpn*I and *Sal*I and cloned into pQE-SA*gapC* to generate plasmid pQE-SA*gapC/Ba* encoding a 2,064 bp ORF encoding a 688 amino acids GapC/B fusion protein with a predicted Mr of 74,385 Da and an isoelectric point (pI) of 5.86.

### **3.3.4. Construction of a Plasmid Expressing the T-cell Plasminogen Activator Signal Sequence.**

To construct a vector expressing the T-cell plasminogen activator signal sequence (Tpa), the 99 bp *Hind*III-*Bgl*II fragment encoding Tpa, obtained by annealing of the complementary oligonucleotides Tpa-1 and Tpa-2 (Table 3.3.1) was ligated to the 4390 bp *Hind*III/*Bgl*II fragment of pBISIA-24. The resultant plasmid was named pBISIA-Tpa (Fig.4.2.2.1). To construct a plasmid expressing the *S. aureus* GapC protein on the cell surface, the 90 bp *Eco*RI/*Bam*HI synthetic Tpa-fragment, obtained by annealing of Tpa-2F and Tpa2-R oligonucleotides (Table 3.3.1), was ligated to the 5310 bp *Eco*RI/*Bam*HI fragment of pBISIA-*gapC*. The resultant plasmid was named pTPA2-*gapC* (Fig.4.2.2. 1). This plasmid possesses an ORF of 1116 bp encoding a Tpa:GapC protein with an estimated Mr of 40,016 Dalton (Da) and pI of 4.0. To construct a plasmid expressing the *S. aureus* GapC/B chimeric protein on the cell surface, the 90 bp *Eco*RI/*Bam*HI synthetic Tpa fragment, obtained by annealing of Tpa-2F and Tpa2-R oligonucleotides (Table 3.3. 1), was ligated to the 6291 bp *Bam*HI/*Eco*RI fragment of

pBISIA-SA*gapC/B*. The resultant plasmid was named pTPA2-*gapC/B* (Fig.4.2.2. 1). This plasmid possesses an ORF of 2112 bp encoding a Tpa:GapC/B protein with an estimated Mr of 76,000 Da and a pI of 5.33. Finally, to construct a plasmid expressing the *S. aureus* GapB protein on the cell surface, the 5331 bp fragment of pTPA2-*gapC/B*, obtained by digestion with *Kpn*I, blunting with T4 DNA polymerase, followed by digestion with *Nhe*I and blunting with the Klenow fragment of DNA polymerase I was self ligated. The resultant plasmid was named pTPA2-*gapB* (Fig.4.2.2. 1). This plasmid possesses an ORF of 1128 bp encoding a Tpa:GapB protein with an estimated Mr of 40,615 Da and a pI of 6.4. Plasmids were purified using the Endofree® Plasmid Maxi Kit (Qiagen, Mississauga, ON, Canada) and the determination of endotoxin content was carried out as before [472].



**Table 3.3.1. Synthetic oligonucleotides used in this study**

Name	Sequence	Description
Sa-1	5' AACATAGGATCCAGCAGCGGCCTGGTGCCGCGC	Forward primer. It binds to the 30-51 bp region of <i>his-gapC</i> in pET-GapC [75]
Sa-2	5' AAATTAGGTACCTTTAGAAAGTTCAGCTAAGTAT	Reverse primer. It binds to the 987-1008 bp region of <i>gapC</i>
Tpa-1	5'-AGCTTGC AATCATGGATGCAATGAAGAGAGGGC TCTGCTGGTGTGCTGCTGTGTGGAGCAGTCTTCGT TTCGGCTAGCCCCGGGTGATAAGGATCCA	Forward oligonucleotide used to construct pBISIA-tpa
Tpa-2	5'-GATCTGGATCCTTATCACCCGGGGCTAGCCGAA ACGAAGACTGCTCCACACAGCAGCAGCACACAGC AGAGCCCTCTCTTCATTGCATCCATGATTGCA	Reverse oligonucleotide used to construct pBISIA-tpa
Tpa-2F	5'-GATCCAGCTTGCAATCATGGATGCAATGAAGAG AGGGCTCTGCTGTGTGCTGCTGTGTGGAGCAG TCTTCGTTTCGGCTAGCCCACG	Forward oligonucleotide used to construct Tpa fusion plasmids
Tpa-2R	5' AATTCGTGGGCTAGCCGAAACGAAGACTGCTCCACA CAGCAGCAGCACACAGCAGAGCCCTCTCTTCATTGCA TCCATGATTGCAAGCTG	Reverse oligonucleotide used to construct Tpa fusion plasmid
Sa-3a	5'-ATGGGTGGTACCGGAAGAATGGTATTACGTAT	Forward primer. It binds to the 40-59 bp region of <i>gapB</i>
Sa-4	5'-GATCCGTCGACGTATTAAGTTCAGCTTACAG	Reverse primer. It binds to the 1010-1026 bp region of <i>gapB</i>
Sa7a	5'AAAAAAGAATTCATGTCAACCAATATTCCAATTAAT CCTAGG	Forward primer used to amplify the <i>S. aureus gapB</i> gene
Sa8a	5'-TTTTTCTCGAGTTAACTGCACTTACA	Reverse primer used to amplify the <i>S. aureus gapB</i> gene
Sa5	5'-AAAAAACCCGGGAGCCATATGGCAGTAAAAG	Forward primer used to amplify the <i>S. aureus gapC</i> gene
Sa10	5'-AAATTACTCGAGTTTAGAAAGTTCAGCTAAGTAT	Reverse primer used to amplify the <i>S. aureus gapC</i> gene
Sa12	5'-CCCTAACTCAACAGGTGCTGCTAAAG	Forward qRT-PCR primer used to amplify part of <i>gapC</i> gene
Sa13	5'-TACAGGAACACGTTGTGCACCACCATC	Reverse qRT-PCR primer to amplify part of <i>gapC</i> gene
SA14	5'-TTCCTACTTCTACTGGTGC GGCGAAA	Forward qRT-PCR primer used to amplify part of <i>gapB</i> gene
SA15	5'-GGTACACGTAATGCCATGCCGTGTAA	Reverse qRT-PCR primer to amplify part of <i>gapB</i> gene
551F	5'-GGCAAGCGTTATCCGGAATT	Forward qRT-PCR primer used to amplify part of 16s rRNA gene of <i>S. aureus</i>
651R	5'-GTTTCCAATGACCCTCCACG	Reverse qRT-PCR primer to amplify part of 16s rRNA gene of <i>S. aureus</i>

The sequences of the primers used to amplify the *S. aureus gapB* and *gapC* genes are shown together with the complementary oligonucleotides encoding the T-cell plasminogen activator secretion signal (Tpa). The underlined nucleotides indicate the recognition site for the restriction endonucleases used in the cloning. In the case of the Tpa-1, Tpa-2, Tpa-2F and Tpa-2-R the respective residual *Hind*III, *Bgl*II, *Eco*RI, and *Bam*HI sites are shown. The primer pairs (Sa12, Sa13), (Sa14, Sa15) and (551F, 651R) are specific for *gapC*, *gapB* and 16s rRNA genes, respectively and were used in qRT-PCR reactions.

### 3.3.5. qRT-PCR

To determine the effects of environmental conditions on *gapC* and *gapB* gene expression, the bovine clinical mastitis *S. aureus* strains SA10, SA12 and SA16 and the *S. aureus* strain RN6390 [205], were grown under different environmental conditions. The conditions tested were pH: tryptic soy broth and whey kept at pH 5, and 7.4 by continuous buffering during bacterial growth; oxygen tension: aerobic, anaerobic, microaerophilic, and high CO<sub>2</sub> levels, for high CO<sub>2</sub>, cultures were grown in CO<sub>2</sub> incubator at 5% CO<sub>2</sub> level; Growth media: Tris minimal glucose media, tris minimal succinate media, trypticase soy broth, and whey. Total RNA was extracted from cultures grown for 7, and 12 hs using RiboPure™ bacterial RNA isolation kit (Ambion Inc, Austin, Texas, USA) following manufacturer's instruction. The *gapC* and *gapB* cDNA were synthesized using *gapC* (sa12, sa13) and *gapB* (sa14, sa15) (Table 3.3.1) specific primer pairs with the SuperScript™ One-Step RT-PCR System (Invitrogen Canada Inc., Burlington, Ont, Canada) following the manufacturer's instruction. The *gapC* and *gapB* gene-expression levels under the above mentioned different environmental conditions were quantified using qRT-PCR (Invitrogen). The 16s rRNA gene of *S. aureus* was amplified with specific primer pairs (551F and 651R) (Table 3.3.1) and used as an internal control for qRT-PCR.

To determine the role of *sar* and *agr* loci on the expression of *gapC* and *gapB* genes in *S. aureus*, total RNA was extracted from wild type strain RN6390 and its isogenic mutants *agrA*, *sarA* and *sar/agr* (double mutant) [468] at the exponential and stationary phases of growth using the RiboPure™ bacterial RNA isolation kit (Ambion Inc. Austin, Texas, USA) following manufacturer's instructions. To determine the expression of *gapC* and *gapB* genes cDNA was synthesized by RT-PCR using specific primers for *gapC* and *gapB*. The cDNA products were used in the qRT-PCR reaction (Invitrogen). The 16s rRNA gene is used as an internal control.

Briefly, amplification was carried out in a total volume of 15µl in 96 well plates using an iCycler qPCR instrument (Bio-Rad). The reaction was carried out using platinum SYBR Green qPCR SuperMix UDG (Invitrogen Canada Inc., Burligton, Ontario) according to the manufacturer's instruction using 3 step amplification cycles with following the parameters: 95 °C 15s, 55 °C 30s, and 72 °C 30s. The amplification of the product was determined by

measuring the amount of SYBR Green I dye incorporated in the PCR product and plotted as fluorescence versus cycle number. The SYBR Green I dye is a double stranded DNA binding dye that emits fluorescence as the double stranded DNA accumulates. The fluorescence intensity increases proportionally to the double stranded DNA concentration [473]. Data from qRT-PCR can be analysed either by relative quantification or absolute quantification methods. Absolute quantification determines the input copy number by relating the PCR signal to a standard curve whereas relative quantification detects the PCR signal of the target transcript in a treatment group to that of another sample such as untreated control. For all conditions tested, cells grown in TSB were considered as the control. The relative difference in *gapC* and *gapB* gene expressions under the above-mentioned conditions was compared to the expression of these genes in TBS under similar environmental conditions. The relative difference in gene expression was calculated as fold change using a formula  $2^{-\Delta\Delta Ct}$  [474], where the Ct was defined as the cycle number at which the first detectable fluorescence increase above the threshold was observed.  $\Delta Ct = Ct$  value of target gene minus Ct value of internal control (Ct value of 16s rRNA). The  $\Delta\Delta Ct = \Delta Ct$  of test minus  $\Delta Ct$  of control. The qRT-PCR reactions were carried out in triplicate and the data showed fold change in mRNA which is  $2^{-\Delta\Delta Ct}$  [474].

### **3.4. Purification of Proteins and Western Blots Analysis**

#### **3.4.1. Whole Cell Protein Extraction Procedure**

Cultures containing *S. aureus* were centrifuged at 4424 X g for 10 min to collect the cells. The cell pellets were suspended in PBS and the suspensions centrifuged as above. The cell pellets were suspended in 5 ml lysis buffer (buffer T1) (100 $\mu$ l of 1M tris-HCl (pH8), 20  $\mu$ l of 0.5M EDTA, 500  $\mu$ l of 10% Triton-X, 100  $\mu$ l of lysozyme at 100 mg/ml final concentration, and sterile water added to bring the final volume to 5 ml). To this solution lysostaphin, RNase I and DNase I were added at concentrations of 100  $\mu$ g/ml, 10 mg/ml and 10 mg/ml respectively. The suspension was incubated at 37 °C for 1 h. The cell debris was removed by centrifugation at 26712 X g for 15 mins and the supernatant was collected in a clean tube.

### 3.4.2. Determination of Protein Concentration in the Whole Cell Lysate

The protein concentration in the whole cell lysate was determined using the Bio-Rad microplate assay protocol following the manufacturer's instruction. Briefly, a series of tubes containing 100, 200, 400, 600, 800, 1000, 1200, 1400, 1600 and 1800  $\mu\text{g/ml}$  of BSA in sterile distilled water was used to construct a standard curve. Since our lysis buffer contained detergent, a 20  $\mu\text{l}$  aliquot of reagent S (Bio-Rad) was added to each ml of reagent A to make reagent A'. All samples were diluted to 1:25 in sterile distilled water. 5  $\mu\text{l}$  of standards and diluted samples were placed into a micro titer plate. 25  $\mu\text{l}$  of reagent A' and 200  $\mu\text{l}$  reagent B were added to each well sequentially. The absorbance was read after 15 min at a wavelength of 750 nm in a spectrophotometer (SpectraMax, Molecular Devices Corporation, Sunnyvale, CA, USA). The total amount of protein in each sample was calculated by comparing the  $A_{750}$  of unknown to the  $A_{750}$  of the standards and using the forecast utility of Microsoft Excel<sup>®</sup> (Microsoft corporation, WA, USA).

### 3.4.3. Recombinant Protein Purification Procedure from *E. coli*

The *E. coli* BL21 (DE3) cells carrying the plasmids containing the *gapB* (pET*gapB*) or *gapC* (pET*gapC*) genes of *S. aureus* BM10 [75] were cultivated to mid-log phase at 37 °C or 25 °C for the strain *E. coli* M15 (pQE-SA*gapC/Ba*) carrying the chimeric plasmid. Expression of genes encoding the 6xHis-GapB, 6xHis-GapC, and 6xHis-GapC/B proteins was induced by the addition of 0.2 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and cells were harvested 3hs post-induction. Purification of the recombinant proteins was carried out by affinity chromatography to Ni-NTA as described before [75]. For immunological analysis, proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Bio-Rad) and probed with anti-GapB or GapC polyclonal antisera as described below. In this text, the 6xHis-GapB, 6xHis-GapC, and 6xHis-GapC/B proteins are referred to simply as GapB, GapC and GapC/B respectively.

#### 3.4.4. Western Blot

SDS polyacrylamide electrophoresis [475] of whole cell total protein extracts was carried out as follows: whole cell extracts containing 20µg total protein were added into eppendorf tubes and the same volume of 2X protein loading dye (100 mM tris HCl, 200 mM dithiothreitol (DTT), 4% SDS, 0.2% bromophenol blue, 20% glycerol) were added to each sample and the mixture was boiled for 5 min and loaded into each lane of a 12% SDS-PAGE. The proteins were separated at 130 volts in a gel apparatus (Bio-Rad). For immunoblotting, proteins were transferred from the gel to the nitrocellulose membrane (Bio-Rad) using a power supply (PowerPac200, Bio-Rad). Briefly, after protein separation the gel was soaked in a transfer buffer (3 g/L tris, 1.41 g/L glycine, 200 ml methanol, distilled water to a final volume of 1000 ml, pH 9) for 5 min. A piece of nitrocellulose matching the size of the gel was cut and soaked in the transfer buffer for 5 min. The nitrocellulose filter was placed on top of the gel and two pieces of pre-cut Whatman filter papers were placed on both the gel and the nitrocellulose sides. This sandwich was placed in between two thick membrane supports and the whole assemble was placed in a holder and into a tank containing pre-chilled transfer buffer. The buffer was kept cold by using a plastic reservoir with ice and a stirrer bar was used to keep buffer circulating. The proteins were transferred at a current of 400 mA for 1 h with stirring. After proteins were transferred to nitrocellulose, the membrane was removed from the apparatus and blocked with 3% bovine serum albumin in 0.1M PBS, 0.05% Tween-20 (pH 7.2) (PBS-T) overnight at 4 °C. After overnight incubation, the membrane was washed three times with PBS-T and incubated with primary antibodies (Rabbit anti-GapB and -GapC sera) as described before [75] at 1/500 dilution for 1 h. After incubation with primary antibodies, the membrane was washed three times with PBS-T and incubated with a secondary antibody conjugated to alkaline phosphatase (Goat anti-rabbit IgG (H+L) (KPL, Gaithersburg, MA, USA). After incubation with the secondary antibody the membrane was washed once with PBS-T and additional once with alkaline phosphatase (AP) buffer (0.1 M tris base and NaCl, each, 0.0051 M MgCl<sub>2</sub>·6H<sub>2</sub>O adjusted to pH 9.5 and autoclaved). The membrane was incubated with 10 ml of AP buffer for 15 min. Finally, the membrane was incubated with nitroblue tetrazolium (NBT) salt and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) 165 µg/ml each, until color developed [476]. The membrane was dried by blotting off excess liquid on Kim-wipes. Developed membranes were scanned with white light fluorescent light and imaging was done using AlphaEase software.

### **3.5. Transfection of MAC-T Cells and Immunohistochemistry**

After 2 days of incubation, confluent monolayers of MAC-T cells were harvested, digested with 0.5 % trypsin, and suspended in culture medium to  $1 \times 10^5$  cells/ml. One ml of the cell suspension was used to seed each of 4 wells of Permaxox® slides and after overnight incubation at 37 °C in 5% CO<sub>2</sub>, the cells were transfected with 1 µg of plasmid DNA using the Lipofectamine-Plus® reagents (Invitrogen) following the manufacturer's recommendations. Transient expression of the *gapB*, *gapC* and *gapC/B* genes was detected by immunohistochemistry (IHC) after incubation at 37 °C for 16 hs. IHC was carried out as follows: cells were fixed with PBS: Acetone (2:3) for 10 min at room temperature. The slide was then washed by three successive immersions in PBS. Antigen-specific antibodies were diluted in PBS-3% horse serum and incubated with the MAC-T cells for 2 hs at room temperature in a humid chamber. After 3 consecutive washes in PBS, the cells were incubated with a 1:2000 dilution of horse anti-mouse IgG conjugated to horse-radish peroxidase for 1 h at room temperature. After washes, the reaction was developed using the ABC and DAB reagent kits (Vector, CA, USA) following manufacturer's instructions.

### **3.6. Mice Immunizations Protocols**

#### **3.6.1. Immunizations with Recombinant Proteins.**

The immune responses to the Gap proteins were determined in C-57BL/6 mice immunized with the purified GapB, GapC and GapC/B proteins. Proteins (20 µg dose) were formulated in 30% VSA3 as adjuvant and administered via the intramuscular route. In all cases, vaccinations were carried out 21 days apart. The first trial consisted of a total of 32 mice divided into 4 groups of eight each. Three groups were immunized with GapB (Group 2), GapC (Group 3) or GapC/B (Group 4) respectively. A placebo group (Group 1) was included. Serum samples were collected at days 0, 21 and 42 and tested by ELISA for the presence of total IgG, IgG1 and IgG2a antibodies against the GapB, GapC and GapC/B proteins using alkaline-phosphatase-conjugated polyclonal antibody against mouse IgG and monoclonal antibodies against mouse IgG1 and IgG2a (KPL, Gaithersburg, MA, USA). Serum titres were calculated by the intersection of least-square regression of  $A_{405}$  versus logarithm of dilution.

The second trial consisted of 40 mice divided into 5 groups of 8 each. The groups consisted of: Group 1 (placebo); Group 2 (GapB); Group 3 (GapC); Group 4 (GapB + GapC) and Group 5 (GapC/B) (Table 3.6.1.1). Humoral immune responses (total IgG, IgG1 and IgG2a) were measured by ELISA assays and titres calculated as described above. After 45 days, the spleens were collected from the immunized animals and lymphocytes were recovered by disruption of the spleen followed by washes and suspension into AIMV culture media. The number of IFN- $\gamma$  and IL-4 producing cells were determined using the ELISPOT assay (section 3.9.2) after seeding  $1 \times 10^6$  cells in tissue-culture wells pre-coated with anti-IFN- $\gamma$  or anti-IL-4 specific antibodies. The proliferation of lymphocytes was determined as described in section 3.9.2.

Table 3.6.1.1. Vaccination of mice with recombinant proteins

<b>Group</b>	<b>Antigen (Ag)</b>	<b>Ag Dose</b>	<b>Adjuvant</b>	<b>Route</b>
1	Placebo	-	30% VSA3	IM
2	GapB	10 $\mu$ g	30% VSA3	IM
3	GapC	10 $\mu$ g	30% VSA3	IM
4	GapC + GapB	10 $\mu$ g (each)	30% VSA3	IM
5	GapC/B	10 $\mu$ g	30% VSA3	IM

The group assignments, vaccine formulation, and route of immunization are described.

### **3.6.2. Immunizations with DNA-Recombinant Proteins Combinations.**

The immune response to the Gap proteins was determined in C-57 BL/6 C mice (5 groups of eight animals each) first immunized with plasmid DNA encoding GapB, GapC and the GapC/B chimera formulated in PBS, 10  $\mu$ g/dose, I.D. route as shown in Table 3.6.2.1. Serum samples were collected at days 0, 28 and 56 and antigen-specific total IgG, IgG1 and IgG2a titres against purified recombinant GapB, GapC and GapC/B proteins were determined as described before [75] using alkaline-phosphatase-conjugated polyclonal antibody against mouse IgG, and monoclonal antibodies against mouse IgG1 and IgG2a. Serum titres were calculated by the intersection of least-square regression of  $A_{405}$  versus logarithm of dilution. At day 97 after the start of the trial, four mice from each group were humanly sacrificed, the spleens were collected and lymphocytes were recovered and the number of cells of IFN- $\gamma$  and IL-4 producing cells were

determined by ELISPOT assay. The proliferation of lymphocytes after stimulation with the recombinant antigens was determined as described in section 3.9.2.

Table 3.6.2.1. Immunization of mice with DNA-recombinant proteins combination

Group	DNA (10 µg)	Protein (20 µg)	Route	Vaccination(day)	Assay (day)
1	pBISIA-tpa	None	ID	0, 28, 56	91
1a	pBISIA-tpa	GapC/B	ID/IM	0, 28, 56, 98*	119
2	pTPA2-gapB	None	ID	0, 28, 56	91
2a	pTPA2-gapB	GapB	ID/IM	0, 28, 56, 98*	119
3	pTPA2-gapC	None	ID	0, 28, 56	91
3a	pTPA2-gapC	GapC	ID/IM	0, 28, 56, 98*	119
4	pTPA2-gapC/B	None	ID	0, 28, 56	91
4a	pTPA2-gapC/B	GapC/B	ID/IM	0, 28, 56, 98*	119
5	None	None	ID	0, 28, 56	77
5a	None	None	ID/IM	0, 28, 56, 98	119

The different immunization groups are shown. The vaccination routes were i.d.: Intradermal and i.m.: Intramuscular. The vaccination column indicates the days of the immunizations and the asterisk denotes the day of the protein boost for groups 1a, 2a, 3a, and 4a. The assay column refers to the day the animals were sacrificed.

### 3.7. Optimizing Antibody Response in the Mammary Gland of Cows

This trial consisted of 40 dairy cows divided in to 5 groups of 8 animals each and immunized with the GapC/B chimera via the subcutaneous (SQ) and intradermal (ID) routes in areas drained by the parotid lymph (PLN) and supramammary lymph (SMLN) nodes (Table 3.7.1).



Table 3.7.1. Vaccination protocol for optimizing antibody response in the mammary gland of cows

Group	Antigen (Ag)	Ag Dose ( $\mu\text{g}$ )	Adjuvant	Adj. Dose	Route/Target	Total volume/dose
1	Placebo	-	VSA3	30%	SQ	2 ml
2	GapC/B	100	VSA3	30%	SQ/PLN	2 ml
3	GapC/B	100	VSA3	30%	SQ/SMLN	2 ml
4	GapC/B	100	VSA3	30%	ID/PLN	1 ml
5	GapC/B	100	VSA3	30%	ID/SMLN	1 ml

### 3.8. Monitoring Duration of Immunity in Cows

This animal trial was designed to determine the duration of the immune response to the best antigen selected from the mouse experiment and to test vaccine formulations containing the adjuvant PCPP (Poly [di(carboxylatophenoxy) phosphazene], synthetic polymer and the immunomodulator CpG ODN 2135 (cytosine-phosphate-guanosine). The trial consisted of 40 dairy cows divided into 5 groups of 8 animals each and immunized via the subcutaneous route (SQ) with 2 ml of vaccine (Table 3.8.1).

Table 3.8.1. Vaccination protocol for monitoring duration of immune response in cows

Group	Antigen (Ag)	Ag Dose ( $\mu\text{g}$ )	Adjuvant	Adj. Dose	Route	Total volume/dose
1	Placebo	-	VSA3	30%	SQ	2ml
2	GapC/B	100	VSA3	30%	SQ	2ml
3	GapC/B	100	CpG	250 $\mu\text{g}$	SQ	2ml
4	GapC/B	100	PCPP	750 $\mu\text{g}$	SQ	2ml
5	GapC/B	100	CpG/PCPP	250/750 $\mu\text{g}$	SQ	2ml

### **3.9. Analysis of Humoral and Cellular Immune Responses**

#### **3.9.1. Enzyme-Linked Immuno-Sorbent Assay (ELISA)**

Proteins were diluted to 1 µg/L in a 50 mM sodium carbonate buffer (1.5 g/L of NaCO<sub>4</sub>, 2.93 g/L NaHCO<sub>4</sub>, to the final volume of 1000 ml with distilled water and pH adjusted to 9.6) and 100 µl of this diluted solution was used to coat each well of a 96-well plate. The plates were covered with a lid, wrapped with saranwrap and placed inside a plastic bag to avoid drying, and incubated overnight for 16 hs at 4 °C. After incubation, the coating solution was removed and plates were washed 5 times with tris-buffered saline (TBS, 0.85% NaCl (w/v), pH7) containing 0.5% (v/v) of Tween 20 (TBS-T) using an automated plate washer. The wells were blocked with 200 µl/well of TBS-0.5% Tween 20 containing 0.03% gelatine (v/v) (TBS-TG) for 2 hs at room temperature. Both serum and whey were serially diluted in four-fold increments from 1:100 to 1:1, 638, 400 and 1:10 to 1:1, 280 respectively in TBS-TG and incubated for 1h. After five washes, alkaline phosphatase-conjugated polyclonal sheep anti-bovine IgG and monoclonal sheep anti-bovine IgG1 and IgG2 (Kirkegaard and Perry laboratories, Gaithersburg, MD, USA) were diluted to 1:5000 in TBS-TG and 100 µl of diluted antibody was added to each well and incubated for 1h. After five washes, 100 µl of freshly prepared para-nitrophenyl phosphate (PNPP, 1 mg/ml, Sigma) substrate solution in DE buffer (10.5 ml of 1 M Diethanolamine, 1 ml of 0.5 M MgCl<sub>2</sub>·6H<sub>2</sub>O and 990 ml of distilled water) was added per well and incubated for 45 min at room temperature. Finally using a microtitre plate reader, the absorbance was read at 405nm with reference at 490 nm. The serum titer was calculated by the intersection of least-square regression of A<sub>405</sub> versus logarithm of dilution.

#### **3.9.2. ELISPOT and Lymphocyte Proliferation Assays**

The production of cytokines (IFN-γ and IL-4) by spleen lymphocytes was determined by ELISPOT assay. Cells were diluted to a density of 1×10<sup>6</sup> cells/well in AIM V media, pre-coated with anti-IFN-γ or anti-IL-4 specific antibodies (BD Biosciences, Sparks, MD, USA). Purified GapB, GapC or GapC/B (200ng/well) was added and incubated overnight at 37 °C in 5% CO<sub>2</sub>. After washes, biotinylated anti-mouse IFN-γ or IL-4 (BD Biosciences, Sparks, MD, USA) were added to the wells and incubated for 2 h. After washes, alkaline phosphatase (AP)-conjugated

streptavidin (Invitrogen, Burlington, Ont, Canada) was added and incubated for 2h. After washes, the alkaline phosphatase (AP) substrate Sigma-Fast (Sigma) was added, incubated 30 min, washed and air dried overnight. The number of IFN- $\gamma$  or IL-4 secreting cells (spots) was determined by counting in a microscope

The proliferation of lymphocytes was determined by seeding  $3 \times 10^5$  cells/well, addition of GapB, GapC or GapC/B (200 ng/well) and incubation for 72 hs at 37 °C in 5% CO<sub>2</sub>. A solution containing 0.4  $\mu$ Ci/well of <sup>3</sup>H-thymidine (Sigma) was added and the cells incubated 18 hs at 37 °C in 5% CO<sub>2</sub>. Cells were harvested and the amount of incorporated <sup>3</sup>H-thymidine was determined in a scintillation counter.

### **3.9.3. Peripheral Blood Mononuclear Cell Isolation and Assessment of Phagocytosis by Phagocytic cells.**

Blood was collected from the jugular vein of sheep (2 X 20 ml) in 0.3% EDTA (Sigma-Aldrich) and centrifuged at 2500 X g for 20 min at room temperature. After this step, the buffy coat was removed and resuspended in 8 ml of PBSA/EDTA (5.4 ml of 0.5 M EDTA (pH 8) to 1000 ml of PBSA, and layered on to 5 ml of Ficoll-Hypaque (GE Health Care Biosciences AB, Uppsala, Sweden) and centrifuged at 3000 X g for 20 min. The supernatant was removed and the pellet of PBMCs was resuspended in 8 ml of PBSA/EDTA and centrifuged at 1200 X g for 10 min at 4 °C. The pellet was resuspended in 10 ml of PBSA/EDTA and centrifuged at 1000 X g for 10 min at 4 °C. The cell pellet was resuspended in 4 ml of cell culture media (minimum essential medium (MEM supplemented with dexamethazone at 1 ng/ml, gentamicin at 50  $\mu$ g/ml, 10% of fetal bovine serum (GIBCO BRL), 1% of L-glutamine (Sigma) and 0.1% of 50 mM solution of 2-mercaptoethanol/) and 40  $\mu$ l was taken and added to 20 ml of Isoton<sup>®</sup>II diluent (Beckman Coulter Inc., Ca, USA) and counted with an automated Coulter particle counter model Z1 (Beckman Coulter Inc., Ca, USA). Cells were diluted to a density of  $1 \times 10^6$  cells/ml.

To determine whether phagocytic cells were phagocytising and removing bacteria. Peripheral blood mononuclear cells (PBMCs) were isolated 48 hs post challenge and incubated in a 6 well tissue culture plates at a density of  $1 \times 10^6$  cells/ml for 2 hs to allow cells to settle down and attach to the plates. After 2 hs of incubation the supernatants were removed and media

with 200 µg/ml gentamicin (Sigma) and 20 µg/ml penicillin (Invitrogen) were added and further incubated for 1 h to kill extracellular bacteria. After incubation, aliquots of supernatants were taken and plated for viable counts and cells were washed gently with growth media without antibiotics. Cells were then lysed by adding 1 ml of distilled water to each well. Lysates were serially diluted and plated for viable counts.

### **3.10. Milk Sample Collection Procedure, Processing and Somatic Cell Count**

#### **3.10.1. Hygienic Milk Collection Procedure.**

Prior to the sampling, quarters of cows and udder halves of sheep were washed and dried. The teat tip was wiped with 70% alcohol and the first few streaks of milk were discarded. About 5-20 ml of milk from sheep and 20-100 ml of milk from cows was collected in sterile 50 ml tubes and 100-500 ml bottles respectively.

#### **3.10.2. Somatic Cell Count**

After collected milk was thoroughly mixed, somatic cells in the milk were fixed by mixing 10 ml of milk with 0.2 ml of the fixative liquid (mixture of 0.02 g of eosin, 9.4 ml of 35% formaldehyde solution and water to 100 ml) [477]. Well mixed samples were kept for 24 hs at 22 °C (room temperature). Then a 0.1 ml of milk test sample was transferred to a tube and diluted with 10 ml of emulsifier electrolyte mixture (125 ml of 96% ethanol, 20 ml of Triton X-100 and 855 ml of 0.9% sodium chloride (saline) solution) [477]. The diluted milk was heated to 80 °C for 10 min and then cooled down to 15-20 °C. The test portion of the milk was transferred to a measuring vessel taking care that no air bubbles were produced. Somatic cells were counted by an automated Coulter particle counter model Z1 (Beckman Coulter Inc., Ca, USA).

### **3.11. Statistics**

Statistical analysis of the humoral and cell-mediated immune responses (One way analysis of variance (ANOVA) and Bonferroni post-test analyses) were performed using GraphPad Prism on logarithmically-transformed data

## 4. EXPERIMENTAL RESULTS

### 4.1. Immune Responses to a GapC/B Chimera and Use as a Component of a Multivalent Vaccine against *Staphylococcus aureus* Mastitis

#### 4.1.1. Introduction

*S. aureus* and coagulase-negative staphylococci are the most prevalent infectious bacteria isolated from bovine mammary gland secretions. Infections with these micro-organisms account for a large percentage of mastitis cases and the economic losses suffered by the dairy industry are in the millions of dollars per year [14]. Combinations of intramammary and systemic treatments or extended therapy to combat this disease results in success rates between 0% and 80% [420, 421] emphasizing the need for alternative control measures. One of these measures is vaccination with killed bacterial cells or bacterial products [442], however with few exceptions they have not always resulted in protection against new infections or do not elicit heterologous protection [439-442, 478]. These findings suggest that isolates from different sources encode distinct products and that a vaccine composed of all the different antigens would be impractical to produce. Recently, a new approach consisting of DNA or DNA/Protein vaccination resulted in partial protection against a *S. aureus* experimental challenge in mice and dairy cows [445, 479]. The DNA vaccines were based on the more conserved epitopes of the fibronectin-binding protein and clumping factor A adhesins of *S. aureus* and elicited both humoral and cellular immune responses. Thus, identification of protective antigens should focus on conserved proteins and on means to elicit Th1 and Th2 responses. Previous work on GapC proteins of environmental streptococci at VIDO resulted in the identification of potential targets for vaccines [480, 481] and the construction of a GapC chimera composed of the *S. uberis* GapC as backbone and non-identical regions of the *S. agalactiae* and *S. dysgalactiae* GapC proteins [481]. The mastitis research was extended to include *S. aureus* and recently our laboratory reported the isolation of surface-located GapB and GapC proteins that have homology to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [75].

In this work, a similar approach of constructing chimeric proteins that can serve as antigens in the formulation of multivalent vaccines was used. A *S. aureus* GapC-GapB chimera

comprised of most of their amino acid residues was constructed and the protein expressed in *E. coli*. The GapC/B chimera was compared to the individual GapB and GapC proteins for its ability to elicit humoral and cellular immune responses in mice. The results indicate that the GapC/B chimera is immunologically indistinguishable from its individual components. In addition, the chimera is able to elicit both humoral and cellular immune responses as determined by the presence of IgG1 and IgG2a in serum and for the secretion of IFN- $\gamma$  and IL-4 by stimulated lymphocytes.

#### **4.1.2. Construction of the Chimeric *gapC/B* Gene**

The goal of this research was to compare the immune response to GapB and GapC proteins of *S. aureus* to the response obtained with the GapC/B chimera. A protein chimera was constructed by PCR amplification of the *gapB* and *gapC* genes of a mastitis-isolate of *S. aureus* described before [75] followed by tandem ligation of the PCR products. The *gapC/B* chimeric gene was cloned in front of a histidine tag for purification and the resultant construct encoded a ca. 74 kDa protein that reacted to polyclonal antibody against GapC (Fig. 4.1.2.1) and GapB (Data not shown). This protein was purified and used in vaccination trials.

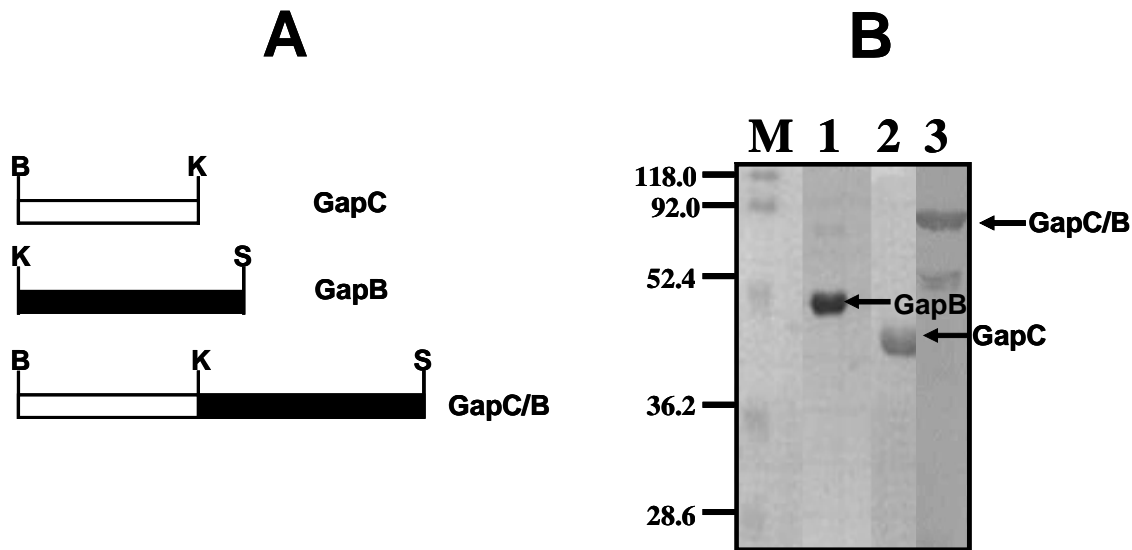


Figure 4.1.2.1. Construction of a *S. aureus* GapC/B chimera.

A: The *gapC* and *gapB* genes were amplified by PCR with primers containing *Bam*HI (B), *Kpn*I (K) and *Sal*I (S) restriction endonuclease sites and cloned into the expression vector pQE-30 digested with *Bam*HI and *Sal*I. B: Composite western blot of *E. coli* extracts containing plasmids expressing 1: GapB; 2: GapC; 3: GapC/B chimera. M: Molecular weight standards. The arrows point to the location of the GapB, GapC and GapC/B proteins.

#### 4.1.3. Humoral Immune Responses to the GapB, GapC and GapC/B Proteins

The use of the GapC/B chimera as a multivalent antigen would be possible only if the immune responses to the protein are comparable to the responses obtained when the individual antigens are used in single or combined vaccine formulations. The chimeric protein GapC/B was constructed, expressed in *E. coli* as a histidine-tagged protein (Fig. 4.1.2.1) and purified by affinity chromatography. The endotoxin content of the recombinant GapB, GapC, and GapC/B proteins was determined to be 4.7, 29.2, and 1.2 EU/ $\mu$ g of protein, respectively. Mice were immunized with the recombinant proteins and their immune responses to the three proteins were determined by measuring total IgG, IgG1 and IgG2a in serum. The results are presented in Fig. 4.1.3.1. Compared to the placebo group, the GapC-specific total IgG, IgG1, IgG2a, titers

significantly increased in mice immunized with GapB ( $p < 0.001$ ) (Fig. 4.1.3.1A) and a weak but significant ( $p < 0.01$ ) cross immune response to GapC was observed as an increase of total IgG and IgG1 GapB titers in mice vaccinated with GapC. Compared to the placebo group, the GapC-specific total IgG, IgG1, IgG2a titers increased significantly ( $p < 0.001$ ) in mice immunized with GapC (Fig. 4.1.3.1.B) and in contrast to GapB-titers in mice vaccinated with GapC, a weak but not significant ( $p > 0.05$ ) total IgG and IgG1 response of GapC was observed in mice immunized with GapB. Finally, compared to the placebo group the total IgG, IgG1 and IgG2a serum titers to GapC/B increased significantly ( $p < 0.001$ ) in mice immunized with GapB or GapC (Fig. 4.1.3.1C). The results of immunization with GapC/B did not completely correlate to the results after vaccination with either GapB or GapC. Compared to the placebo group, the total IgG and IgG1 serum titers to GapC/B increased significantly in mice immunized with GapC/B ( $p < 0.001$ ) albeit they were 10-fold lower (on average) than the titers against the individual proteins; however the IgG2a titers were not significantly different ( $p > 0.05$ ) to the placebo group (data not shown). It has been determined that the reason for the differences in serum titers was a partially degraded GapC/B protein and thus we prepared a new batch of the GapC/B protein and stored it in the presence of protease inhibitors and used it in a second mice immunization experiment to confirm our serology findings. This time we also included a vaccination group that received the individual GapB and GapC or the chimeric GapC/B proteins and the results are presented in Fig 4.1.3.2. Compared to the placebo group, immunization with GapB-containing vaccines resulted in a significant increase ( $p < 0.001$ ) of the total IgG titers in the Groups 2, 4, and 5 (GapB, GapB + GapC or GapC/B groups, respectively) while immunization with GapC (Group 3) resulted in higher but not significantly different GapB titers ( $p > 0.05$ ) than the placebo group (Group 1) (Fig 4.1.3.2A) but significantly lower ( $p < 0.001$ ) than the GapB, GapB + GapC or GapC/B groups. The IgG1 titers to GapB were significantly different to the placebo group ( $p < 0.001$ ) except in the GapC-immunized mice ( $p > 0.05$ ) and not significantly different ( $p > 0.05$ ) between the groups that received any form of GapB (Groups 2, 4, and 5). Like the GapB total IgG titers, the IgG1 levels in the GapB, GapB + GapC or GapC/B groups were significantly higher than the titers in the animals that received GapC (Group 3) Fig. 4.1.3.2A). Immunization with GapB resulted in significantly higher ( $p < 0.001$ ) GapB-IgG2a titers in the animals immunized with any form of GapB (Groups 2, 4, and 5) compared to the placebo and GapC groups (Groups 1 and 3 respectively). Unexpectedly, the GapB-IgG2a titers in mice immunized with GapB + GapC were



significantly lower than the titers in the GapC/B group ( $p < 0.001$ ) (Fig 4.1.3.2A) which might suggest interferences between the GapB and GapC proteins when not in the same molecule.

Compared to the placebo group, immunization with any form of GapC (Groups 3, 4, and 5) resulted in significantly higher GapC-total IgG titres ( $p < 0.001$ ) (Fig. 4.1.3.2B). Contrary to the GapB titers in the GapC-immunized group (Group 3) (Fig. 4.1.3.2A), the GapC titres in the GapB-vaccinated group (Group 2) were also significantly different from the placebo ( $p < 0.001$ ), suggesting a stronger cross-reaction between GapC antibodies against GapB. As observed before (Fig 4.1.3.2A), there seemed to be interference between the GapB and GapC proteins in the GapB + GapC group (Group 4) since the total IgG titers to GapC were lower ( $p < 0.05$ ) in this group compared to the titers in the GapC/B group (Group 5) (Fig.4.1.3. 2B). The IgG1 titers to GapC in groups 2, 3, 4, and 5 were significantly different than the placebo group ( $p < 0.001$ ), however contrary to the total IgG levels, the GapC-IgG1 titers in the GapB-vaccinated group were lower ( $p < 0.05$ ) than the titers in the GapC-immunized and the GapC/B-vaccinated groups ( $p < 0.001$ ). Moreover, the GapB IgG1 titers were higher ( $p < 0.05$ ) in the GapC/B-vaccinated group compared to the GapB + GapC group (Groups 5 and 4 respectively). The GapC-IgG2a titers were significantly higher than the placebo group in all the vaccinated animals ( $p < 0.001$  for Groups 2, 3, and 5;  $p < 0.01$  for group 4, respectively) (Fig. 4.1.3.2B). There were no significant differences ( $p > 0.05$ ) between the IgG2a titers in the GapB-, GapC- and GapC/B-immunized groups but like before, the titers in the GapB + GapC-vaccinated group (Group 4) were significantly lower than the other vaccinated groups ( $p < 0.001$ ), suggesting again interferences between these two proteins when not in the same molecule.

Compared to the placebo group, immunization with GapC/B resulted in a significant increase ( $p < 0.001$ ) of total IgG titers against GapC/B not only in group 5 but in the groups that received GapB (Group 2), GapC (Group 3) and the GapB + GapC (Group 4) combination (Fig 4.1.3.2C). A similar picture was observed with the GapC/B-IgG1 titers (Fig.4.1.3. 2C). The GapC/B IgG2a titers in the GapB-immunized group were not different than the titers in the GapC/B group ( $p < 0.05$ ). Unexpectedly, the IgG2a-GapC/B titers in the GapC-immunized mice were lower than the titers in the GapB-vaccinated animals although this difference was not significant and as observed before for the IgG2a titers against GapB and GapC in the GapB +

GapC-immunized animals (Fig. 4.1.3.2A and B), the IgG2a-GapC/B titers in the GapB + GapC group (Group 4) were significantly lower than the titers in the GapB (Group 2)-, GapC (Group 3)-, and GapC/B (Group 5)-immunized mice ( $p$  values:  $<0.001$ ;  $<0.05$  and  $<0.001$  respectively) (Fig. 4.1.3. 2C).

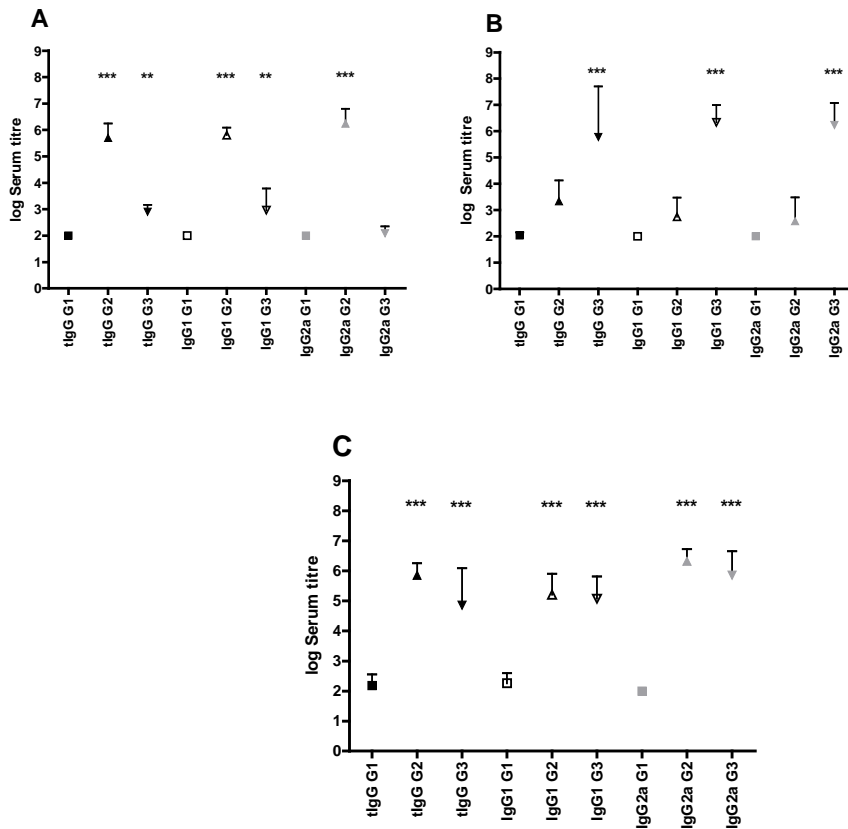


Figure 4.1.3.1. Humoral total IgG, IgG1 and IgG2a titers against GapB, GapC and GapC/B.

Humoral immune responses to GapB, GapC, and GapC/B (trial I), titers were measured 42 days after the first immunization. The immunoglobulin isotypes and vaccine groups are indicated on the X-axis while the titers to GapB, GapC, and GapC/B are shown in the panels A, B, and C respectively. The symbols are mean + S.D. of the titers. The asterisks above the columns represent statistically different titer levels between groups.

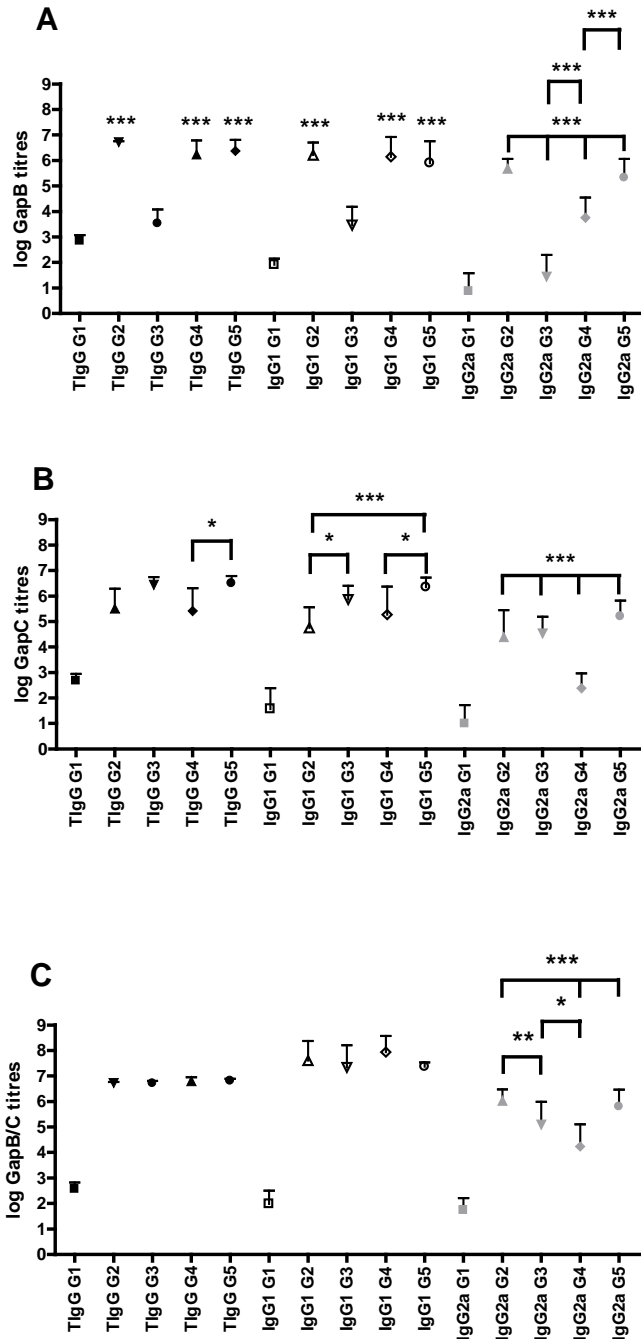


Figure 4.1.3.2. Humoral immune responses to GapB, GapC and GapC/B.

Humoral immune responses to GapB, GapC and GapC/B (Trial 2), titers were measured 42 days after the first immunization. The immunoglobulin isotypes and vaccine groups are indicated on the X-axis while the titers to GapB, GapC, and GapC/B are shown in the panels A, B, and C respectively. The symbols are means + S.D. of the titers. The asterisks above the columns represent statistically different titer levels between groups.

#### 4.1.4. Cellular Immune Responses to the GapB, GapC and GapC/B Proteins

Cytokine expression assays were performed after stimulation with the GapB, GapC or GapC/B proteins. Compared to the placebo group, stimulation with GapB resulted in significantly higher IL-4 expression in mice vaccinated with GapB or GapB + GapC ( $p < 0.01$  and  $p < 0.05$  respectively) while GapC stimulation was significantly higher ( $p < 0.05$ ) than the placebo group only in mice vaccinated with GapB + GapC (Fig 4.1.4.1A). Stimulation with GapC/B in animals vaccinated with it resulted in higher numbers of spots as compare to the placebo group, however, due to the variation in the assay, this difference was not significantly different ( $p > 0.05$ ). Secretion of IFN- $\gamma$  after stimulation with GapB was significantly higher than the placebo group in GapB- and GapC/B- immunized animals and different from the placebo group in GapB- and GapC/B-immunized animals only (Groups 2 and 5) ( $p < 0.001$ ). Stimulation with GapC resulted in significantly higher IFN- $\gamma$  expression in mice immunized with GapB, GapC/B (groups 2 and 4;  $p < 0.01$  and  $p < 0.001$  respectively) (Fig. 4.1.4.1B). The levels of IFN- $\gamma$  expression in the groups vaccinated with GapC or GapB + GapC were similar to the level in the GapB-vaccinated group, however due to the variability of the assay these numbers were not significantly different from the placebo group ( $p < 0.05$ ). Stimulation with GapC/B promoted significant IFN- $\gamma$  secretion in mice vaccinated with GapB (Group 2,  $p < 0.001$ ) and GapC/B (Group 5,  $p < 0.001$ ) only (Fig. 4.1.4.1B).

Activation of cell-mediated immunity was also evaluated by measurement of the lymphoproliferation responses to the recombinant proteins. Stimulation with GapB induced significant blastogenesis only in mice immunized with GapB ( $p < 0.001$ ) (Fig.4.1.4.1C) while GapC significantly induced proliferation of splenocytes of mice immunized with GapB (Group 2,  $p < 0.05$ ), GapB + GapC formulation (Group 4,  $p < 0.001$ ) and with the GapC/B chimera (Group 5,  $p < 0.001$ ) (Fig.4.1.4.1C). Finally, stimulation with the GapC/B chimera resulted in significant levels ( $p < 0.001$ ) of lymphocyte proliferation in the GapB-, GapB + GapC- and GapC/B-immunized groups (Groups 2, 4, and 5 respectively) (Fig 4.1.4.1C).

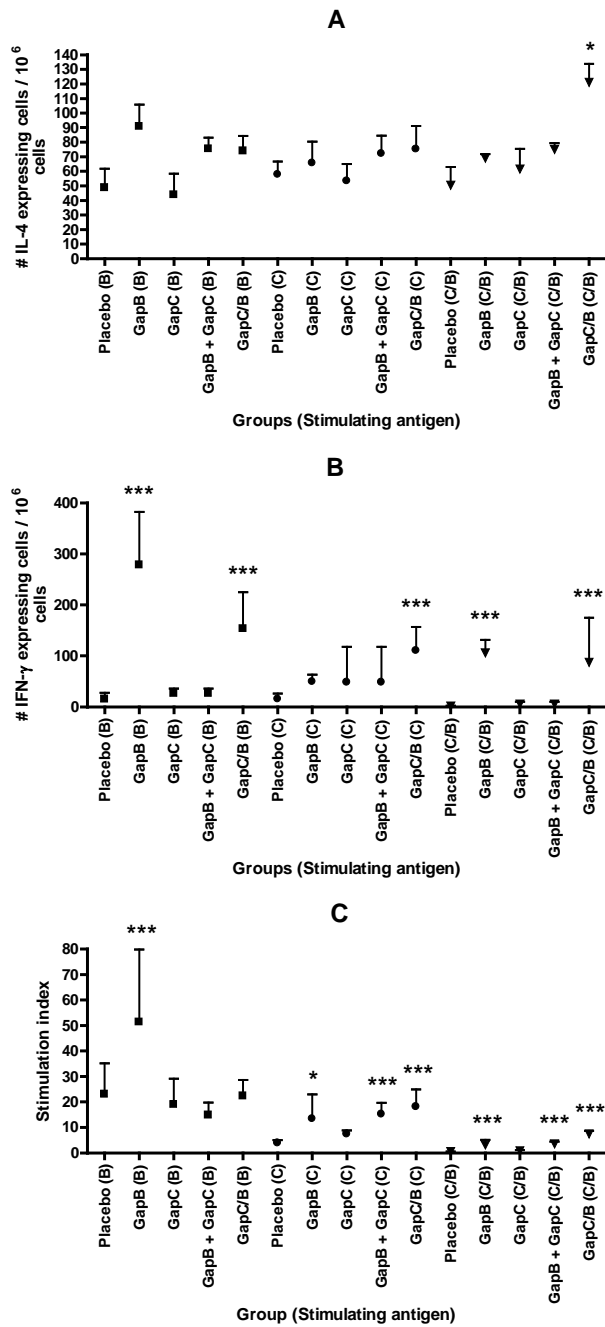


Figure 4.1.4.1. Cellular immune responses to GapB, GapC and GapC/B.

The production of IL-4, IFN- $\gamma$ , and stimulation index of splenocytes with GapB, GapC, or GapC/B is shown. (A) IL-4 expression; (B) IFN- $\gamma$  expression; (C) Lymphocyte proliferation. Significantly different IL-4, IFN- $\gamma$  or stimulation indexes between vaccine groups are shown by asterisks. The production of IL-4, IFN- $\gamma$ , and stimulation index of lymphocytes with GapB, GapC or GapC/B is shown. A: IL-4 expression; B: IFN- $\gamma$  expression; C: Lymphocyte proliferation. Significantly different (\*:  $p < 0.05$ ; \*\*\*:  $p < 0.001$ ) IL-4, IFN- $\gamma$  or stimulation indexes between vaccine groups are shown by asterisks. See text for more explanation.

#### 4.1.5. Antibody Recognition of Native GapC and GapB Proteins

The recombinant forms of GapB, GapC or GapC/B contains the 6 X histidine tag for purification by affinity chromatography [75] (Fig 4.1.2.1.) and as expected, sera of mice immunized with these proteins reacted with purified recombinant proteins on the ELISA assays (Fig 4.1.3.1 and 4.1.3.2). To determine if these immune responses were detected to the GapB and GapC portion of the recombinant proteins and not to the histidine tag, we tested whether the mice immune sera would also react with native GapB and GapC extracted from the surface of several isolates of *S. aureus*. We prepared extracts from the human isolates of *S. aureus* Sa2 and from bovine mastitis isolates *S. aureus* Sa4, Sa5, Sa7, Sa10 (source of the *gapB*, and *gapC* genes; [75] and Sa12 and performed ELISA assays using pooled mice anti-GapC/B sera as the primary antibody anti-mouse IgG1 and IgG2a as secondary antibodies. The results (Fig. 4.1.5.1A) showed that pooled mice immune sera recognized the native proteins as judged by the IgG1 and IgG2a titers. Dairy cows are the ultimate target for a vaccine containing GapC and GapB as one of its components. To assess whether these proteins were recognized by the immune system of cows naturally exposed to *S. aureus*, we carried out a western blot against the GapB proteins using serum from a cow with a history of chronic mastitis due to *S. aureus*. The results (Fig. 4.1.5.1B) indicated that the serum was able to recognize the recombinant proteins.

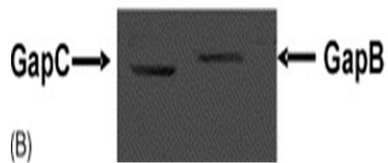
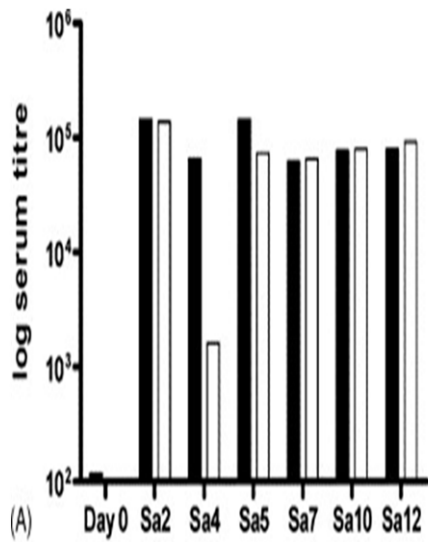


Figure 4.1.5.1A and B. Antibody recognition of GapC and GapB proteins.

(A) GapC/B titers against cell surface extracts. The mouse GapC/B IgG1 (black bars) and IgG2a (white bars) titers against the surface extracts are presented. The day 0 (pre-immune serum) was used as negative control. (B) Western blot of purified GapB and GapC proteins with serum from a cow with chronic mastitis due to *S. aureus*.

#### 4.1.6. Discussion

Mastitis caused by *S. aureus* remains the most costly disease for dairy farmers. Although antibiotic therapy and culling of infected animals are the most effective approaches to control of the disease, the success rate of treatment against *S. aureus* infections varies considerably. When the treatment costs and losses due to reduced milk production, discarding of the milk and culling of the animals are factored, it is obvious that new approaches to control the disease such as vaccination are needed. The family of proteins with GAPDH activity are conserved at the DNA and protein sequence level and have been shown to be potential components of vaccines against a variety of bacterial, mycotic, and parasitic infections [77, 480-484]. The identification of GapB and GapC on the surface of *S. aureus* [75] allowed us to construct a GapC/B chimera for producing a single multivalent antigen. The protein was expressed in *E. coli* and it reacted to specific GapC (Fig 4.1.2. 1) and GapB antisera (data not shown). As to the rationale of using a chimera composed of GapB and GapC, we felt that while the role of GapC as a protective antigen is well documented, the addition of another surface-expressed protein could increase the potential role of the vaccine to protect against *S. aureus* infections.

The use of the GapC/B chimera as a single antigen would be possible only if the immune responses to the protein are comparable to the responses obtained when the individual antigens are used in single or combined vaccine formulations. On the first mice immunization trial, we observed significant total IgG, IgG1, and IgG2a antibody levels against the GapB and GapC proteins on all vaccinated groups (Fig 4.1.3.1). Immunization with GapC/B however, results in approximately 10 fold less total IgG and IgG1 titers on average as compared to immunization with GapB or GapC and practically undetectable IgG2a titers (data not shown). We determined that the amount of GapC/B protein used was lower than originally estimated and this might have had a moderate effect on the antibody titers. Nevertheless, the IgG2a titers against GapB and GapC are suggestive of a cellular immune response and thus we decided to repeat the mice immunization experiment and to measure cell mediated and humoral immune responses.

Immune responses to GapB, GapC and GapC/B were determined and with minor exceptions, the results indicate that in general, there are no differences between the total IgG and



IgG1 titres when the GapB or GapC or GapC/B proteins are used compared to titers resulting from immunization with a mixture of the GapB + GapC proteins (Group 4). It would be logical to expect no differences in the serum titers against the three proteins; however the animals that received the GapB + GapC mixed formulation developed a comparably lower IgG2a response to the individual proteins and the GapC/B chimera (although significantly higher than the placebo group) (Fig 4.1.3.2). This suggest interference between these two proteins, since degradation of the vaccine components was discounted after analysis by Western blots of the remainder of the vaccine after it was sent back to the laboratory (data not shown).

The GapB and GapC proteins share 47% identity [75], thus some degree of immune-cross-reactivity between these proteins was expected. Overall we detected cross-immune responses but not to the levels observed with the cognate antigens. In the first trial, GapC-vaccinated animals apparently developed significantly higher antibodies titers against GapB compared to the placebo group but the opposite was not true (Fig 4.1.3.1A and B). In the second trial, mice that received the GapB protein only (Group 2) developed a significant response to GapC (Fig 4.1.3.2B) but the opposite was not true (Fig 4.1.3.2A). This apparent contradiction between trials can be explained by comparing the titers to GapB in the placebo groups in the two trials. Comparison of GapB titers between the placebo groups indicates that the titers in the first trial are approximately 10 fold lower than the titers in the second trial (Fig 4.1.2.1A and 4.1.3.2A) perhaps due to different mice lots used for the trials. The lower placebo titers might account for the statistical significance ( $p < 0.01$ ) observed between the GapB titer of mice of the placebo and Group 3 in the first trial (Fig 4.1.2.1A).

Published evidence of the role of proteins with homology to GAPDH as protective antigens and the results obtained here after vaccination with GapC (*i.e.* high serum titres against GapB and GapC) would suggest that a vaccine solely composed of this protein would suffice and the GapC/B chimera would not be needed. Previous results with the GapC protein of *S. dysgalactiae* suggest otherwise. Immunization of dairy cows with *S. dysgalactiae* GapC (closely related to *S. uberis* GapC) resulted in a lack of protection against a *S. uberis* experimental challenge [480] indicating that despite close relationships, immunization with proteins sharing a high degree of homology is not a guarantee of cross-protection.

More supporting evidence for the addition of GapB to a vaccine formulation comes from the results obtained here when markers of cellular immune responses were analyzed. In general, GapB is better than GapC in inducing IL-4 expression (Fig 4.1.4.1). This was somewhat unexpected since the total IgG and IgG1 serum titers to both proteins are not different between all the groups (except the placebo). Formulation that contains GapB (either as individual protein or as part of the GapC/B chimera) are able to stimulate IL-4 production in cells obtained from mice immunized with GapB or GapB + GapC (Fig 4.1.4.1A) while stimulation with GapC is only significant in mice vaccinated with the GapB + GapC combination (Fig 4.1.4.1A), perhaps due to cross-reaction. Stimulation with GapC/B also shows a higher number of IL-4 secreting cells than the placebo in groups 2, 4, and 5 (Fig 4.1.4.1A); however in none of these groups is the number of IL-4 producing cells significantly different from the placebo. This is also unexpected since vaccination with GapC/B resulted in an increase of serum titers in all the test groups (Fig. 4.1.3.2). In our hands the IL-4 ELISPOT assay seems to be more variable and sometimes the antibody titers do not correlate with the number of IL-4-secreting cells [485]. GapB also performs better than GapC at inducing IFN- $\gamma$  expression and promoting blastogenesis (Fig. 4.1.4.1B and C) despite showing similar GapB- or GapC-specific IgG2a titers (Fig. 4.1.3.2A and B). Since the GapC-vaccinated mice show high GapB-IgG2a levels (Fig. 4.1.3.2B) it is not totally surprising to detect IFN- $\gamma$ -secreting cells or a proliferative response in cells from mice from group 2 after stimulation with GapC. The effects of IFN- $\gamma$  and blastogenesis on cells from mice vaccinated with GapC/B followed by stimulation with the cognate antigen can also be attributed to the GapB portion of the protein since significant levels of IFN- $\gamma$  and stimulation indexes are seen in cells from the GapB- and GapC/B-vaccinated animals (Fig. 4.1.4.1B and C). The possibility of contaminating endotoxin affecting the results was discarded since GapC (which had the highest endotoxin amounts) does not induce production of IL-4 or IFN- $\gamma$  and neither promotes splenocyte proliferation as well as GapB or GapC/B (Fig. 4.1.4.1). Although it appears that GapB is a better antigen in several laboratories including ours, GapC in GAPDH-related sequences were shown to be protective [77, 480-484]. As to whether to use a mixture of GapB + GapC or a chimera protein containing approximately 50% residues from GapB and 50% residues from GapC, we believe that GapC/B is a better choice. The animals that received the GapB + GapC mixed formulation developed a comparably lower IgG2a response compared to

the individual proteins (Fig. 4.1.3.2) and this correlates with low number of IFN- $\gamma$ -secreting cells collected from these mice after stimulation with any of the three antigens (Fig. 4.1.4.1B). Thus a chimeric protein composed of GapB and GapC sequences not only will reduce the cost of antigen production but also has a greater potential to offer protection than the individual proteins. Although we cannot completely discard the contribution of the histidine tags in the recombinant proteins to the immune responses in mice, we are confident that it is negligible since mice sera was able to recognize proteins extracted from the surface of several *S. aureus* strains, including a human isolate (Fig. 4.1.5.1A). Moreover, serum from a cow naturally exposed to *S. aureus* reacts with the recombinant GapB and GapC proteins (Fig. 4.1.5.1B), again suggesting that the histidine tag plays no role in the development of antibodies to these two proteins. More importantly, recognition of these proteins by the target species indicates that they are available to the cow's immune system in the native state, i.e., as components of the surface-located proteins of *S. aureus*.

A comparison of the amino acid sequence of the GAPDH proteins of *S. aureus* to the bovine and mouse GAPDH proteins indicate that overall there is 43% identity between the *S. aureus* and bovine GAPDH and 44% between the mouse and *S. aureus* GAPDH (data not shown). A close inspection at the homologous regions between these proteins reveals that most of the identity lies near or at the GAPDH active site (residues 145 to 223 of *S. aureus* GapC). We concede that the GapC protein of *S. aureus* as a whole might not be the ideal candidate for vaccination since the potential exists for developing an auto-immune disease after vaccination with GapC. There is no information available however as to whether this problem exists in cows chronically exposed to *S. aureus* or to other bacteria that possess GAPDH-related sequences. Nevertheless it might be necessary to construct derivatives lacking undesirable regions before the ultimate vaccine prototype can be tested.

Immunization of dairy cows represents an economical alternative to control mastitis caused by *S. aureus* and experimental vaccines containing various antigen preparations have been tried, however with ambiguous results [439-442, 478]. The use of conserved epitopes in a DNA/Protein vaccination approach partially protected against an experimental challenge with *S. aureus* [445, 479] which suggests that the use of conserved antigens as part of a single protein molecule as a vaccine component is a viable alternative. Thus, we believe that our approach of

using GapB and GapC protein-sequences assembled in a GapC/B chimera as a potential target for developing of a vaccine to protect dairy cows from mastitis caused by *S. aureus* would increase the arsenal available to combat this economically important disease.

## **4.2. DNA-Protein Immunization against the GapB and GapC Proteins of a Mastitis Isolate of *Staphylococcus aureus***

### **4.2.1. Introduction**

Vaccination with DNA has been proposed as an alternative to using bacterins or recombinant proteins as the antigenic components of a vaccine [107, 454]. The advantages and pitfalls of the use of DNA for vaccination were recently reviewed [486]. DNA or DNA/protein vaccination against *S. aureus* resulted in partial protection against a *S. aureus* experimental challenge in mice and dairy cows [445, 479, 487]. The DNA vaccines were based either on the penicillin-binding protein (PBP2a) encoded by the *mecA* gene or on more conserved epitopes of the fibronectin-binding protein and clumping factor A adhesins of *S. aureus* and elicited both humoral and cellular immune responses.

In this study the GapB, GapC and GapC/B antigens were tested as candidates for DNA vaccination by constructing plasmids expressing these proteins. The humoral and cell-mediated immune responses in mice were determined after immunizations with either plasmids alone or a combination of priming with plasmid DNA followed by a boost with the recombinant proteins.

### **4.2.2. Construction of Plasmids Encoding a Protein Export Signal and Protein Expression on the Surface of MAC-T Cells**

The tests to determine the immune responses to the *S. aureus* proteins encoded by derivatives of pBISIA-24 were conducted first. This plasmid has been used successfully to elicit immune responses to viral antigens [469]. The plasmids pBISIA-*gapB*, pBISIA-*gapC* and pBISIA-*gapC/B* were used to immunize mice and serum and cell-mediated immune responses were determined. The results indicated that vaccination with these plasmids was not enough to mount a detectable immune response to the *S. aureus* antigens (data not shown). The reason for

the low immune response could be that the *S. aureus* proteins were present in the cytoplasm of the cells and no or few antigen molecules were expressed on the cell surface where they could be detected by antigen-presenting cells. To express the proteins on the cell surface, a pair of complementary oligonucleotides (Table 3.3.1) encoding the protein-export sequence from the T-cell plasminogen activator (Tpa) was inserted in front of the *gapB*, *gapC* and *gapC/B* genes resulting in the plasmids pTPA2-*gapB*, pTPA-*gapC* and pTPA2-*gapC/B* respectively (Fig. 4.2.2.1). We transfected MAC-T cells and used immunohistochemistry (IHC) to assess the expression of the GapB, GapC and GapC/B antigens on the cell surface. The results (Fig.4.2.2.2) indicated that these antigens were expressed on the cell surface of MAC-T cells.

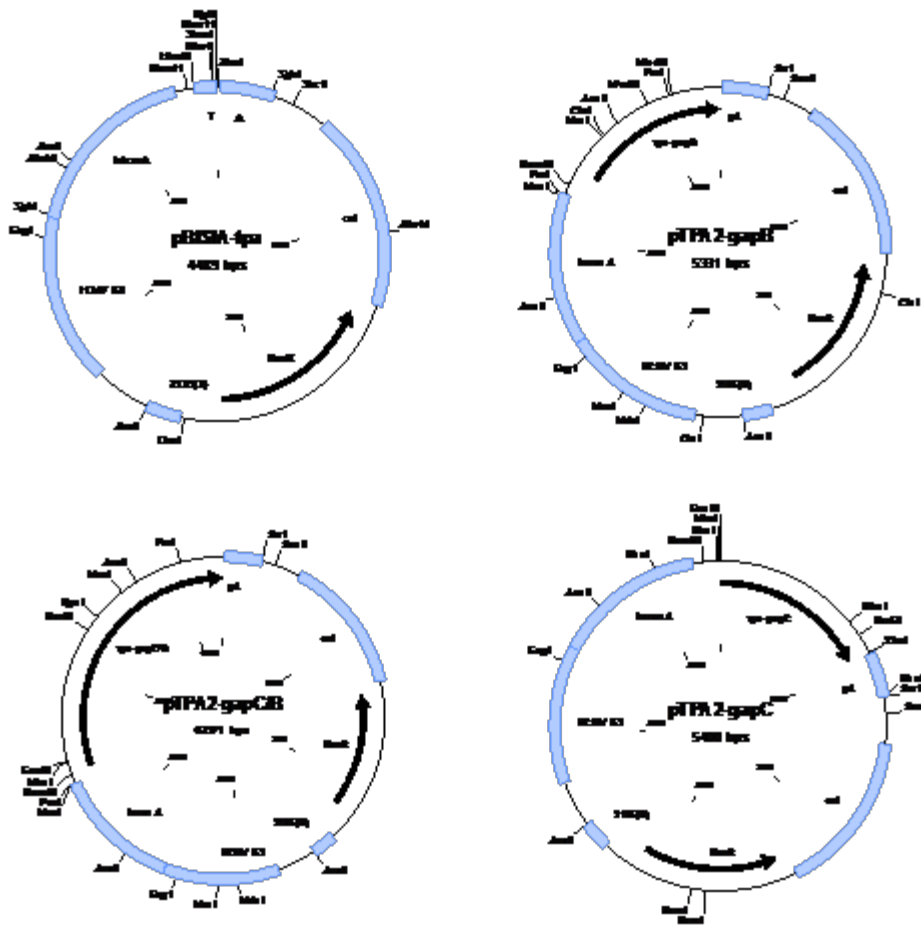


Figure 4.2.2.1. Construction of plasmids encoding protein export signal.

To express the proteins on the cell surface, a pair of complementary oligonucleotides (Table 3.3.1) encoding the protein-export sequence from the T-cell plasminogen activator (Tpa) was inserted in front of the *gapB*, *gapC* and *gapC/B* genes resulting in the plasmids pTPA2-*gapB*, pTPA2-*gapC* and pTPA2-*gapC/B* respectively. A vector carrying only signal sequence (pBISIA-tpa) also constructed.

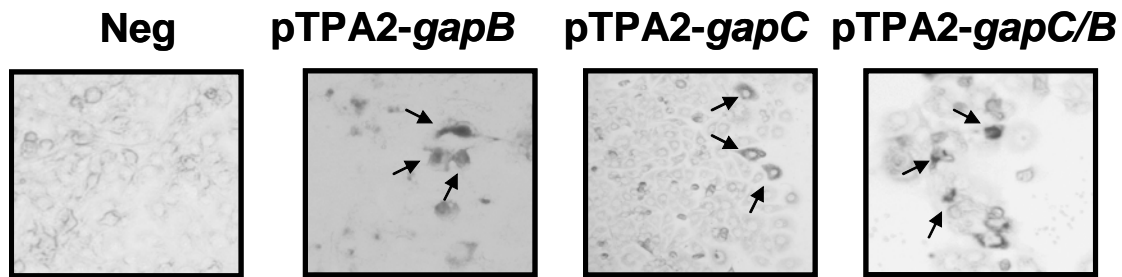


Figure 4.2.2.2. Expression of proteins on the surface of MAC-T cells.

The plasmids pTPA2-*gapB*, pTPA2-*gapC* and pTPA2-*gapC/B* encoding the *gapC*, *gapB*, and *gapC/B* genes respectively, were transfected into MAC-T cells and the presence of the proteins was detected by immunohistochemistry (IHC) as described in section 3.5. Negative MAC-T cells with vector alone, pTPA2-*gapB*, pTPA2-*gapC*, and pTPA2-*gapC/B*: MAC-T cells expressing GapB, GapC and GapC/B, respectively. The arrows point to the location of MAC-T cells expressing the proteins.

#### 4.2.3. Immune Responses to DNA Vaccination

To determine the immune response to the GapB, GapC and GapC/B antigens encoded by pTPA2-*gapB*, pTPA-*gapC* and pTPA2-*gapC/B* respectively, mice were immunized with plasmid DNA by the intradermal route (Table 3.6.2.1). The immune responses were first assessed by measuring antibody titers to the proteins followed by ELISPOT and cell-proliferation assays. The results of the ELISA tests indicated that there was no significant total IgG, IgG1 and IgG2a serum response to the three antigens (data not shown). When the cell-mediated immune response was measured by ELISPOT and lymphoproliferation tests in four mice from each group, the results showed low production of IL-4 (Fig. 4.2.3.1A) and IFN- $\gamma$  (Fig. 4.2.3.1B) and low stimulation indexes (Fig. 4.2.3.1C) in all of the vaccinated groups with respect to the control group.

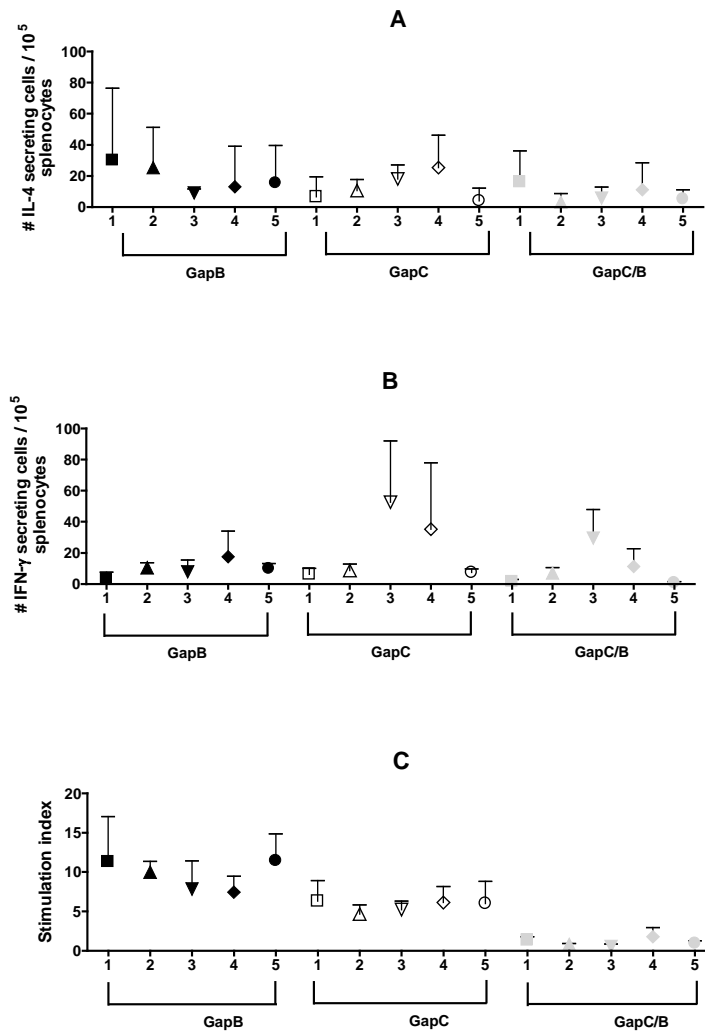


Figure 4.2.3.1. Cellular immune responses to GapB, GapC and GapC/B after immunization with plasmid DNA.

The production of IL-4, IFN- $\gamma$ , and stimulation index of splenocytes with GapB, GapC or GapC/B is shown. A: IL-4 expression; B: IFN- $\gamma$  expression; C: Lymphocyte proliferation. The symbols are mean + S.D. The vaccine groups are indicated on the X-axis while the recall antigens are indicated by the brackets below the X-axis. Group 1 animals received pBISIA-TPA, while groups 2- 4 were immunized with pTPA2-gapB, pTPA2-gapC and pTPA2gapC/B, respectively. Group 5 (placebo) received a PBS solution.



#### 4.2.4. Immune Responses to DNA-Protein Vaccination

The low humoral and cell-mediated responses obtained after immunization with plasmid DNA indicated that these plasmids alone were not adequate to mount an immune response. A previous report indicated that mice immunization with plasmid DNA encoding the CTLA-ClfA hybrid protein followed by a boost with ClfA resulted in a higher humoral response compared to the response in mice vaccinated with the plasmid alone [479]. Therefore, the remaining four mice from each group were boosted with the recombinant proteins as indicated in Table 3.6.2.1. The serum total IgG, IgG1, and IgG2a levels as well as the number of IL-4-, IFN- $\gamma$ -secreting cells and lymphoproliferation indexes were measured after stimulation with the respective recall antigens.

A boost immunization with GapB (Fig. 4.2.4.1A) resulted in significant increases of total IgG titers in the group primed with pTPA2-*gapB* and boosted with GapB (Group 2a) compared to groups primed with pTPA2-*gapC* and boosted with GapC (Group 3a,  $p < 0.05$ ), primed with pTPA2-*gapC/B* and boosted with GapC/B (Group 4a,  $p < 0.01$ ) and placebo (Group 5a,  $p < 0.001$ ) while no significant differences were observed between the serum titers of groups 2a and 1a which received a GapC/B boost after vector only immunization. GapB-specific IgG1 titers significantly increased in groups 1a and 2a with respect to the placebo group (Fig. 4.2.4.1A). The titers in group 2a were also significantly different from the GapB antibody levels in groups 3a ( $p < 0.05$ ) and 4a ( $p < 0.01$ ) respectively. The GapB-specific IgG2a titers were elevated in all the vaccinated groups (Fig. 4.2.4.1A) but these titers were different ( $p < 0.05$ ) in group 2a with respect to the placebo group 5a.

A different result was observed when the GapC-specific serum titers were measured after a boost immunization with GapC. Vaccination with this protein resulted in a significant increase ( $p < 0.001$ ) only in the animals primed with pTPA2-*gapC* and boosted with GapC (group 3a, Fig. 4.2.4.1B). Similar results were observed when the GapC-specific IgG1 titers were measured. In this case GapC-specific IgG1 titers were significantly elevated ( $p < 0.001$ ) in group 3a with respect to groups 1a, 4a and 5a (Fig. 4.2.4.1B). The IgG1 GapC-specific serum titers in group 3a were also significantly different ( $p < 0.05$ ) to the titers in mice primed with pTPA2-*gapB* (Group

2a). In addition, the IgG1 GapC-specific titers in animals that received a GapC/B boost after vector only immunization (Group 1a) were significantly different ( $p<0.01$ ) than the placebo, (Group 5a) titers. Finally, GapC-specific IgG2a titers were also significantly different in group 3a with respect to group 1a ( $p<0.001$ ), group 2a ( $p<0.05$ ) and groups 4a and 5a ( $p<0.01$ ) respectively.

A boost immunization with GapC/B resulted in significantly elevated total IgG in animals of groups 1a ( $p<0.001$ ), 2a ( $p<0.001$ ), 3a ( $p<0.001$ ) and 4a ( $p<0.01$ ) with respect to the placebo group 5a (Fig. 4.2.4.1C). In addition the group 2a GapC/B-specific total IgG was significantly different ( $p<0.01$ ) than the titers in group 3a. A similar picture was observed with the GapC/B-specific IgG1 titers. In all the vaccinated groups, a significant increase of the serum titers was observed in groups 1a, 2a, and 3a ( $p<0.001$ ) and in group 4a ( $p<0.01$ ) compared to the placebo group (Group 5a). The titers in group 4a however, were significantly lower than the titers in groups 1a, 2a and 3a ( $p$ -values  $<0.05$ ,  $p<0.01$ , and  $<0.05$ , respectively). As observed with the total IgG titers against GapC/B, the IgG2a titers in groups 1a, 2a and 3a were significantly different than the titers in group 4a ( $p$ -values  $<0.05$ ;  $<0.01$  and  $<0.05$ ), respectively.

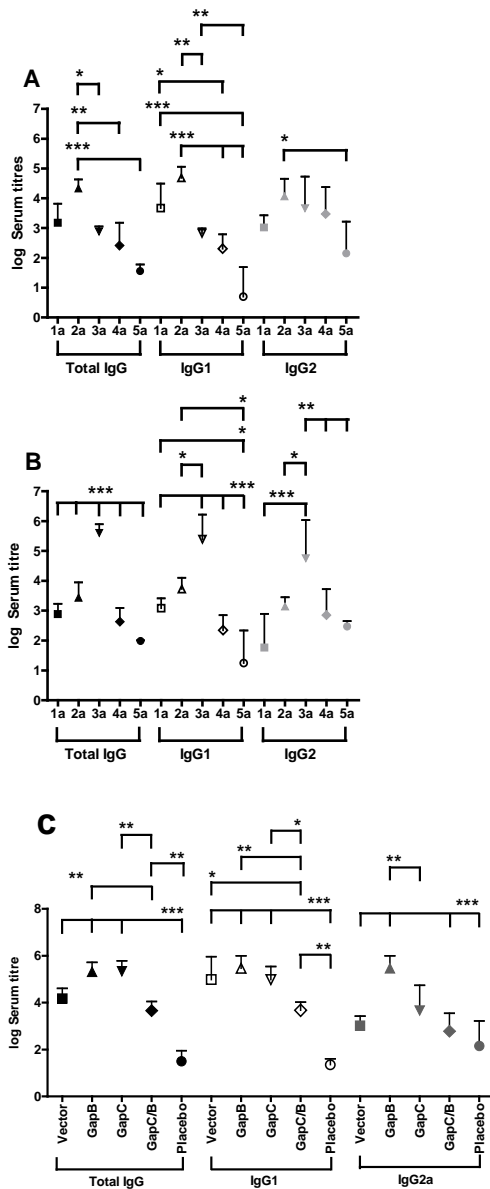


Figure 4.2.4.1. Humoral immune responses to GapB, GapC and GapC/B after vaccination with plasmid DNA and boost with recombinant proteins.

Titers to GapB, GapC, and GapC/B (measured 119 days after the first immunization) are shown in panels A-C, respectively. The vaccine groups are indicated on the X-axis. The symbols are mean + S.D. of the titers. The asterisks above the columns represent statistically different titers level between groups. Group 1a animals received a boost of GapC/B, while groups 2a, 3a, 4a were boosted with GapB, GapC and GapC/B, respectively. Group 5a (Placebo) received a PBS solution.

#### 4.2.5. Cell-mediated Responses in Animals Boosted with the Recombinant Proteins

Contrary to the results obtained when mice were immunized with plasmid DNA only (Fig. 4.2.3.1) increased cell-mediated immune responses were observed in animals primed with DNA and boosted with proteins (Fig. 4.2.5.1). Stimulation with GapB resulted in an increase of the number of IL-4-secreting cells in all immunized animals (Fig. 4.2.5.1A). The number of IL-4-secreting cells of animals of the group 2a (Primed with pTPA2-*gapB* and boosted with GapB) was significantly higher ( $p<0.001$ ) than the placebo group 5a only. The number of IL-4 secreting cells of the animals primed with pTPA2-*gapC* and boosted with GapC (Group 3a) was significantly higher than the number of cells of group 1a (Primed with pBISA-TPA and boosted with GapC/B,  $p<0.001$ ), the group 2a (Primed with pTPA2-*gapB* and boosted with GapB,  $p<0.01$ ), group 4a (Primed with pTPA2-*gapC/B* and boosted with GapC/B,  $p<0.001$ ), and group 5a (Placebo,  $p<0.001$ ). In addition, a significant number of IL-4-secreting cells were observed in the group 1a compared to the placebo group 5a ( $p<0.05$ ). Priming with pTPA2-*gapC/B* followed by a boost immunization with GapC/B did not significantly increase the number of IL-4-secreting cells after stimulation with GapB. Stimulation with GapC (Fig. 4.2.5.1A) resulted in a significant increase in the number of IL-4-secreting cells in group 3a (Primed with pTPA2-*gapC* and boosted with GapC) compared to groups 4a (Primed with pTPA2-*gapC/B* and boosted with GapC/B,  $p<0.01$ ) and 5a (Placebo,  $p<0.01$ ) and to group 1a (Primed with pBISA-TPA and boosted with GapC/B,  $p<0.05$ ). Priming with pTPA2-*gapB* or pTPA2-*gapC/B* followed by boost immunizations with GapB or GapC/B (Groups 2a and 4a respectively) did not significantly increase the number of IL-4-secreting cells compared to the placebo group 5a after stimulation with GapC. Stimulation with GapC/B resulted in a significant increase of the IL-4-secreting cells of all vaccinated groups with respect to the placebo group 5a (1a:  $p<0.001$ ; 2a:  $p<0.05$ ; 3a:  $p<0.001$  and 4a:  $p<0.001$ ). No differences however were detected amongst the different vaccinated groups.

The numbers of IFN- $\gamma$ -secreting cells were determined after stimulation with GapB, GapC or GapC/B and the results are presented in Figure 4.2.5.1B. Stimulation with GapB resulted in an increase of the number of IFN- $\gamma$ -secreting cells in all immunized animals with respect to the number of cells of the placebo group 5a ( $p<0.001$ ). As observed before for the IL-

4-secreting cells, the number of IFN- $\gamma$ -secreting cells of group 3a (Primed with pTPA2-*gapC* and boosted with GapC) was significantly higher than the number of cells of group 4a (Primed with pTPA2-*gapC/B* and boosted with GapC/B,  $p < 0.01$ ) and higher (Albeit not significant,  $p > 0.05$ ) than the number of cells of group 2a (Primed with pTPA2-*gapB* and boosted with GapB). The number of IFN- $\gamma$ -secreting cells of group 3a however, was significantly higher than the number of cells secreting IFN- $\gamma$  of group 4a (Primed with pTPA2-*gapC/B* and boosted with GapC/B,  $p < 0.05$ ). Stimulation with GapC resulted in a significant increase of the IFN- $\gamma$ -secreting cells of group 3a animals (Primed with pTPA2-*gapC* and boosted with GapC) compared to groups 1a, 2a, 4a and 5a ( $p < 0.001$ ) and no other differences were found amongst the groups. As observed before for the number of IL-4-secreting cells, stimulation with GapC/B also resulted in a significant increase ( $p < 0.001$ ) of the IFN- $\gamma$ -secreting cells in all the vaccinated animals with respect to the placebo group 5a (Fig. 4.2.5.1B). In this case, however, cells from group 2a (Primed with pTPA2-*gapB* and boosted with GapB) showed a significant decrease in the number of IFN- $\gamma$ -secreting cells when compared to the number of cells of the groups 3a (Primed with pTPA2-*gapC* and boosted with GapC,  $p < 0.001$ ) and 4a (Primed with pTPA2-*gapC/B* and boosted with GapC/B,  $p < 0.01$ ) respectively.

The results of the lymphoproliferation assays after stimulation with GapB, GapC or GapC/B are presented in Figure 4.2.5.1C. Stimulation with GapB resulted in significant proliferation indexes in cells obtained from animals of group 2a (Primed with pTPA2-*gapB* and boosted with GapB) compared to the indexes in groups 1a (Primed with pBISIA-TPA and boosted with GapC/B,  $p < 0.001$ ), 4a (Primed with pTPA2-*gapC/B* and boosted with GapC/B,  $p < 0.001$ ) and 5a (Placebo,  $p < 0.001$ ) but not when compared to the stimulation index in group 3a (Primed with pTPA2-*gapC* and boosted with GapC,  $p > 0.05$ ). In addition, cells from group 3a showed significant differences ( $p < 0.001$ ) in the stimulation index when compared to the placebo group 5a only. The stimulation index of the cells of groups 1a and 4a did not show a significant increase with respect to group 5a (Fig. 4.2.5.1C). A comparable result was obtained when GapC was used as the recall antigen. In this case, the stimulation index was significantly higher ( $p < 0.001$ ) in group 3a animals (Primed with pTPA2-*gapC* and boosted with GapC) when compared to the indexes in groups 1a, 2a, 4a, and 5a (Fig. 4.2.5.1C). As seen before for GapB, addition of GapC to the cells of group 2a (Primed with pTPA2-*gapB* and boosted with GapB)

resulted in a stimulation index significantly higher than the index observed in the placebo group 5a ( $p<0.01$ ) only. Addition of GapC/B resulted in significant stimulation indexes in cells from all vaccinated groups compared to the placebo group 5a (Fig. 4.2.5.1C) although these indexes were smaller than the ones observed after stimulation with either GapB or GapC (Note the difference on the scale of Fig. 4.2.5.1C). Compared to the placebo group 5a, the highest stimulation indexes were observed in groups 2a (Primed with pTPA2-*gapB* and boosted with GapB,  $p<0.001$ ), 3a (Primed with pTPA2-*gapC* and boosted with GapC,  $p<0.001$ ), followed by the group 1a (Primed with pBISIA-TPA and boosted with GapC/B,  $p<0.01$ ) and finally group 4a (Primed with pTPA2-*gapC/B* and boosted with GapC/B,  $p<0.05$ ). No differences however were detected amongst the different vaccinated groups.

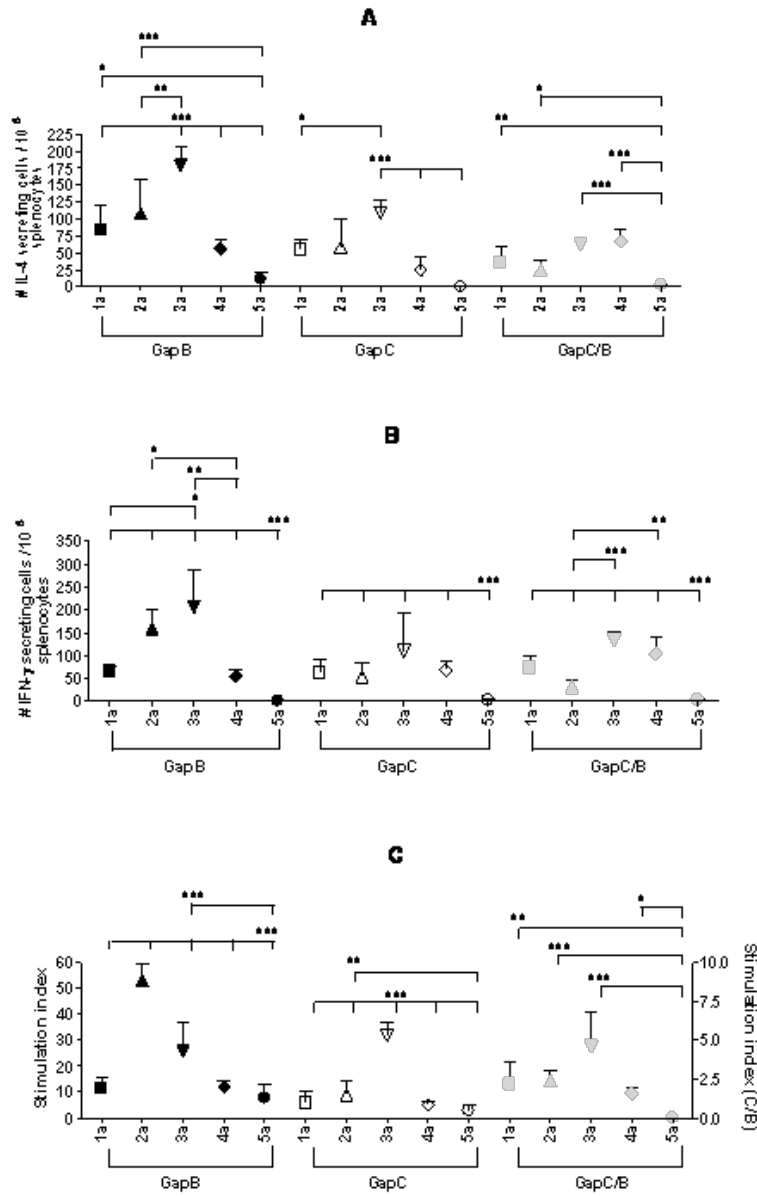


Figure 4.2.5.1. Cellular immune responses to GapB, GapC, and GapC/B after vaccination with plasmid DNA and boost with recombinant proteins.

The production of IL-4, IFN- $\gamma$ , and stimulation index of splenocytes with GapB, GapC or GapC/B are shown. A: IL-4 expression; B: IFN- $\gamma$  expression; C: Lymphocyte proliferation. Note the different scale used for the stimulation indexes after addition of the GapC/B recombinant protein. The symbols are means + S.D. The asterisks above the columns represent statistically different values between groups. The vaccine groups are indicated on the X-axis while the recall antigens are indicated by the brackets below the X-axis. The vaccine groups are the same as of Fig. 4.2.4.1.

#### 4.2.6. Discussion

In the last few years it has been proposed that immunization with plasmid DNA might be a viable alternative to the more traditional approach of using proteins or whole cell lysates as the antigenic component of a vaccine (reviewed in [486]). The objective of the present study was to compare the quantity and quality of the immune response to *S. aureus* antigens encoded by plasmid DNA. This work was started by constructing plasmids encoding the GapB, GapC and GapC/B genes of *S. aureus* SA10 [75, 472] using a vector possessing eukaryotic promoter and enhancer regions together with 3 tandem copies of the CpG ODN 2135 which had previously been used to increase cell-mediated immune responses in bovine species [469, 488, 489]. Since there is experimental evidence suggesting that low levels of surface-exposed or secreted antigens results in sub-optimal immune responses [489, 490], these plasmids were modified by addition of a synthetic segment of DNA encoding a derivative of the T-cell plasminogen activator protein export sequence (Table 3.2.6.1) to promote expression of antigens on the cell surface [490]. Using the modified plasmids, expression of recombinant GapB, GapC and GapC/B on the surface of transfected MAC-T cells was detected (Fig.4.2.2.2) but no significant humoral and very low cell mediated immune responses were observed even after three rounds of immunizations (data not shown, and Fig. 4.2.3.1, respectively). Despite the low immune responses to the antigens encoded by the plasmids, an additional boost with the recombinant proteins was conducted to determine if this would enhance the quality and quantity of the immune responses in mice.

In a previous trial, immunizations with GapB or GapC resulted in significant increases of total IgG, IgG1 and IgG2a serum titers against the cognate antigen and the recombinant GapC/B chimera, while immunization with GapC/B resulted in significant serum titers against the three proteins. In addition, GapC titers in GapB-immunized animals were also elevated suggesting cross-reaction between these antibodies [472]. In addition, GapC titers in GapB-immunized animals were also elevated suggesting cross-reaction between these antibodies [491]. In the trials reported here, the GapB- and GapC-specific total IgG, IgG1 and IgG2a titers are significantly higher than the placebo group in the groups immunized with pTPA2-*gapB* or pTPA2-*gapC* and boosted with GapB or GapC, respectively, and antibody-cross-reaction between the two groups



was observed (Figs. 4.2.4.1A and B). In addition, the total IgG and IgG1 serum titers against GapC/B increased in all the groups (Except placebo) regardless of the antigen used for priming or boosting (Fig. 4.2.4.1C) a result similar to what was reported before [472]. In the trial reported here however, only the group primed with pTPA2-*gapC* and boosted with GapC (Group 3a, Fig. 4.2.4.1C) showed a significant increase of GapC/B-specific IgG2a titers. Priming with pBISIA-TPA followed by a boost with GapC/B (Group 1a) also results in an increase of the total IgG, IgG1 and IgG2a titers against all the recombinant proteins compared to the placebo group (Fig. 4.2.4.1). This finding is not totally unexpected. Due to the time period between the protein boost and assay days (21 days, Table 3.6.2.1) one would expect an immune response to the GapC/B chimera and as observed before [491], we would also anticipate a response against the individual proteins. Compared to the titers in group 1a, the GapB-specific titers in group 2a are higher and the GapC-specific titers in group 3a are significantly higher whereas the GapC/B-specific titers in group 4a are not statistically different from the titers in group 1a (But significantly different than the placebo group). These results suggest that the humoral immune responses observed are solely due to the boost with the recombinant proteins.

The results of the tests designed to check for cell-mediated immunity present a similar picture to the results observed before and after two immunizations with GapB and GapC (Fig. 4.2.5.1). The number of antigen-specific IL-4-, IFN- $\gamma$ -secreting cells and stimulation indexes were all significantly elevated with respect to the placebo group. As observed before for the serum titers (Fig. 4.2.4.1), the best IL-4 and IFN- $\gamma$  responses are obtained after priming with pTPA2-*gapC* and boosting with GapC (Even in animals vaccinated with any form of GapB, presumably due to cross-antigenic reaction), followed by the responses obtained in the group 2a. The stimulation index in splenocytes isolated from mice primed with either pTPA2- *gapB* or pTPA2-*gapC* and boosted with cognate antigens shows significant differences after stimulation with their respective recall antigens compared to the stimulation index obtained in cells from animals primed with pBISIA-TPA and boosted with GapC/B. The animals primed with pTPA2-*gapC/B* and boosted with GapC/B however show lower cell-mediated responses to GapC/B although the number of IL-4 and IFN- $\gamma$  secreting cells from the group 4a animals is slightly higher, but not significant, than the number of cells in group 1a (Fig. 4.2.4.1A and B). Due to a limit in the number of animals, we were unable to include groups of mice primed with the vector

alone and boosted with GapB and GapC and thus we could not assess the the effect of a single immunization with the protein antigens versus priming with DNA and boosting with the antigens. Thus, taking all these results together it appears that the immune responses to GapB, GapC and GapC/B are due to the boost vaccination with the recombinant antigens.

The lower immune responses obtained in mice after priming with the plasmid-encoded antigens could be due to several factors including codon bias (Although the *S. aureus* antigens were detected on the surface of MAC- T cells (Fig.4.2.2.2)), uptake of antigens by APC or the type of CpG present on the plasmid. CpG oligonucleotides are small DNA molecules that belong to the family of pathogen-associated molecular patterns (PAMP). CpG motifs bind to the toll-like receptor (TLR9) molecules and upon recognition, cell-signalling pathways are induced resulting in a pro-inflammatory response and predominantly a Th-1-like immune response (reviewed in [492]. Analysis of the TLR9 receptor sequences shows greater homology between cattle and human than cattle and mice receptors. This degree of homology results in different CpG motifs able to stimulate mice and bovine leukocytes (reviewed in [493]. In addition to showing different TLR9-receptors specificities, cattle appear to diverge from the Th-1/Th-2 paradigm in that the majority of T cell clones appear to fall into the Th-0 type expressing both IL-4 and IFN- $\gamma$  [494] whereas in mice, the Th-1- and Th-2- mediated responses are often mutually exclusive and reciprocally regulated by the cytokines they secrete in vivo [495, 496]. Despite the differences, in both species the Th-1 and Th-2 responses have been linked to the expression of specific cytokines for example IFN- $\gamma$  is linked to IgG2 in cows and IgG2a and IgG3 in mice and IL-4 to IgG1 and IgE production in both species [497, 498]. Immune response against other *S. aureus* antigens were obtained after DNA immunization in mice [479, 487] and cows [445, 446]. Virulence studies performed in mice indicate that immunization with the MecA protein protects the animals from a bacterial challenge [487] and vaccination with plasmid DNA encoding the clumping factor A (CfIA) resulted in a balanced Th-1/Th-2 immune response; however it did not protect mice from an intraperitoneal challenge although the immunized serum reduced the ability of *S. aureus* to bind to fibrinogen [479]. In cows, a balanced Th1/Th2 immune response was obtained after immunization with plasmid DNA encoding the *S. aureus* protein A and followed by a boost with the recombinant protein [446], vaccination with protein alone proved to be less effective in mounting an immune response when compared to a DNA priming/protein boost

regime and no information on protection against a challenge was provided. Another study indicates that priming the immune system with a mixture of two plasmids, one encoding the tandem fibronectin-binding repeats D<sub>1</sub>D<sub>3</sub>, an internal ribosomal entry site and the CflA protein and the second encoding bGM-CSF followed by a boost with the recombinant D<sub>1</sub>D<sub>3</sub> and CflA proteins offered partial protection against a *S. aureus* challenge of dairy cows [445]. Using viral antigens immunization with plasmids possessing a similar backbone to the one reported here was effective to elicit a strong humoral and cell-mediated immune response in cattle [490]. Plasmids encoding the CpG ODN 2135, HCMV IEI promoter, intron A region, Tpa secretion signal and viral antigens have been shown to elicit significant Th-1/Th-2 balanced immune responses in cattle [489, 490, 499-502] and with lesser activity in mice [503]. In this work, we conclude that adding genes encoding *S. aureus* surface located antigens to these plasmids allows us to extend their range of use although it remains to be determined whether these plasmids will be effective in mounting an immune response against the *S. aureus* GapB and GapC proteins in dairy cows.

### **4.3. Optimizing the Immune Response in the Mammary Gland of Cows.**

#### **4.3.1. Introduction**

In large animals, protection from intramammary infections is mainly achieved by the systemically induced inflammatory and adaptive immune responses that recruit immune cells and molecules into the mammary gland through multiple steps that requires expression of several mediators and receptors to achieve transfer of these immune effectors from blood to the mammary gland. Moreover, quick recruitment with increased numbers of immune cells (especially neutrophils) and molecules (example IgG2) are required to either prevent or control intramammary infection. The human mammary gland is part of the mucosal immune system where immunization through mucosal surfaces will induce protection in the mammary gland whereas in large animals it is not clearly known whether the mammary gland is part of mucosal immune system. For bovine mastitis, it is well known that phagocytic neutrophils together with pathogen specific opsonising antibodies (mainly IgG2) are the major immune effectors in the mammary gland [251]. The recognition of invading pathogens and prompt inflammatory responses that recruit neutrophils into the mammary gland are important immune responses required for prevention of intramammary infections [504-506]. Dendritic cells, blood vessel endothelial cells, mammary epithelial cells, milk macrophages and T and B lymphocytes of the peripheral immune system also play important roles in the local inflammatory response helping to recruit opsonising antibodies and neutrophils from blood into milk for rapid phagocytic clearance of opsonised pathogens [251]. Milk and its components have significant diluting and blocking effects on recruited immune effectors. To avoid dilution of immune effectors in milk during intramammary infection, intramammary immune effectors should be recruited in higher numbers. In healthy udders the major cell types are macrophages in dry and lactating cows, and neutrophils in colostrum. Lymphocytes account for 10-27% of cells during lactation [507]. The numbers of lymphocytes are high in the secretion of involuted udders but their numbers are less during the periparturient period [508-510]. In the normal population of udder milk,  $\alpha\beta$  T-lymphocytes are higher than  $\gamma\delta$  T-lymphocytes. The  $\alpha\beta$  T-lymphocytes are predominantly CD8+ and show the phenotype of memory cells [245, 360, 511]. Milk macrophages are the first cells to encounter invading mastitis pathogens and they are also reported to be involved in phagocytosis [512].

As a means to improve bovine intramammary immunity we hypothesize that enhancing local intramammary immunity by targeting vaccine delivery to the supramammary lymph node through an appropriate route of injection will strengthen the intramammary immunity by increasing local production of immune effectors especially IgA production in the supramammary lymph node. Immunoglobulin A is predominant in milk of humans and rodents, whereas IgG is the dominant immunoglobulin in milk of ruminants and pigs [513]. It has been shown that IgA producing plasma cells home to secretory epithelial cells after an initial stimulus with their antigen in the mucosal lymphoid tissue [343]. We hypothesize that local injection in the area drained by the supramammary lymph node might increase IgA production in the mammary gland more efficiently than from IgA producing B-lymphocytes that home to the mammary gland from distant gut associated lymphoid tissue. In this objective, we tested both the route of injection (Subcutaneous versus intradermal) and the site of injection (Parotid lymph node area for systemic response versus supramammary lymph node for local response) in the mammary gland. This hypothesis was tested by two intradermal (ID) and subcutaneous (SQ) vaccinations with GapC/B chimeric antigen 21 days apart, into the area drained by the supramammary lymph node (SMLN). The trial design is shown in Table 3.7.1. As controls, immunizations in the area drained by the parotid lymph node (PL) were carried out. The placebo group (Group 1) (Table 3.7.1) was injected only with adjuvant (VSA3). This comparison of target sites was carried out in dairy cows during the involution period. This is the time when local production of IgA has the greatest impact on the total Ig levels in mammary secretions. The total IgG, IgG1, IgG2 and IgA levels were determined in serum and mammary secretions.

#### **4.3.2. Humoral Immune Response in Serum and Mammary Gland of Cows**

Compared to the placebo group, subcutaneous vaccination in the supramammary lymph node area resulted in significantly higher serum ( $p < 0.01$  for all immunoglobulins isotypes indicated) and milk ( $p$ -values  $< 0.05$ ,  $< 0.01$ ,  $< 0.05$ , and  $< 0.01$ ) GapC/B total IgG, IgG1, IgG2 and IgA titers respectively. Similarly intradermal vaccination in the supramammary lymph node area resulted in significantly higher serum ( $p$ -values  $< 0.001$ ,  $< 0.01$ ,  $< 0.001$  and  $< 0.001$ ) and milk ( $p$ -

values <0.001, <0.001, <0.001 and <0.01) GapC/B IgG, IgG1, IgG2 and IgA titers respectively. Compared to the placebo, intradermal vaccination with GapC/B chimeric protein in the parotid lymph node area induced significantly higher serum ( $p$ -values <0.001, <0.001, <0.01 and <0.001) and milk ( $p$ -values <0.001, <0.01, <0.01 and <0.05) GapC/B total IgG, IgG1, IgG2 and IgA titers respectively. On the other hand, subcutaneous injection of GapC/B near the parotid lymph node areas did not stimulate significantly higher ( $p>0.05$ ) GapC/B total IgG, IgG2 and IgA titers in milk and serum; however, it induced significantly higher ( $p<0.05$ ) GapC/B IgG1 titers in milk and serum; however, it induced significantly higher ( $p<0.05$ ) GapC/B IgG1 titers in both milk and serum (Fig. 4.3.2.1).

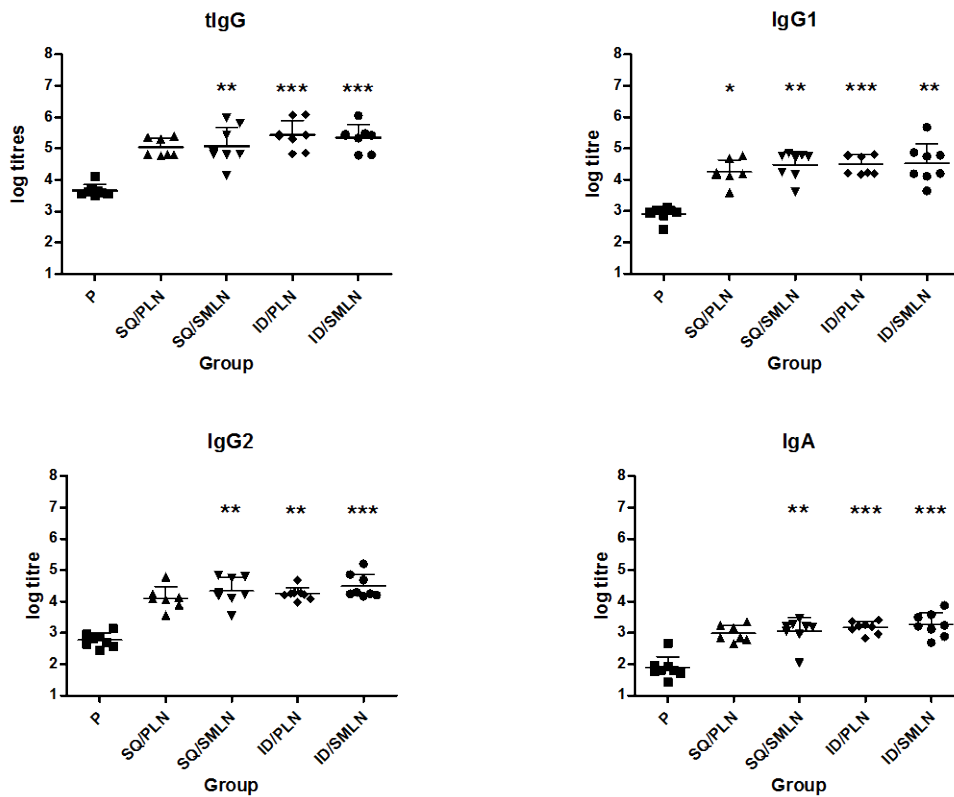


Figure 4.3.2.1. Serum titers to GapC/B.

Humoral immune responses to GapC/B were measured 42 days after the first immunization. The vaccine groups are indicated on the X-axis while the serum titers of total IgG, IgG1, IgG2, and IgA are shown in their respective panels. Each symbol represents one animal in the group. The bars across the symbols are mean + SD of the titers. The asterisks above the column represent statistically different titer levels (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ) between groups.

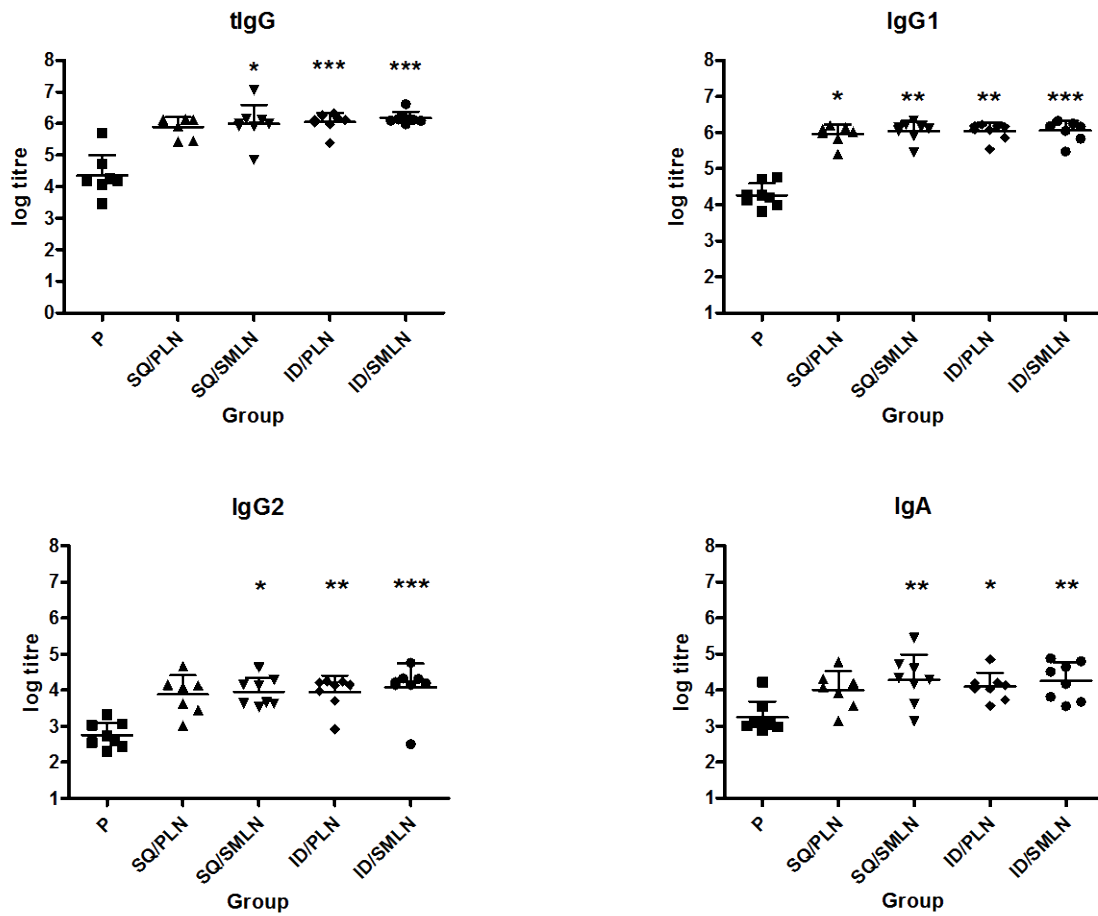


Figure 4.3.2.2. Milk titers to GapC/B.

Anti-GapC/B titers in milk were measured 42 days after the first immunization. The vaccine groups are indicated on the X-axis while the titers of total IgG, IgG1, IgG2 and IgA are shown in their respective panels. Each symbol represents one animal in the group. The bars across the symbols are mean + S.D. of the titers. The asterisks above the column represent statistically different titer levels (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ) between groups.

### 4.3.3. Discussion

In this trial both subcutaneous and intradermal immunizations with the GapC/B protein near the supramammary lymph node and intradermal immunization near parotid lymph node areas resulted in significantly increased serum and milk titers of total IgG, IgG1, IgG2, and IgA in all vaccinated groups as compared to the placebo. The GapC/B IgG1 serum and milk titers were significantly higher in all vaccinated group as compared to the placebo group. These results indicated that local vaccination at the area drained by supramammary lymph nodes has a small advantage over vaccination at the distant anterior parotid lymph node area. Both intradermal and subcutaneous injections at supramammary lymph nodes induced significantly higher GapC/B IgG, IgG1, IgG2 and IgA titers in both milk and serum. Intradermal vaccination at the parotid lymph node area also induced significantly higher GapC/B IgG, IgG1, IgG2 and IgA titers in both milk and serum; however, subcutaneous vaccination in the parotid lymph node area resulted in significantly high IgG1 titers in serum and milk. The IgG immunoglobulin is synthesized by lymph node-resident B-cells, collected from serum and transcytosed through the endothelial and epithelial cell layers by a prolactin-sensitive mechanism [514] whereas IgA immunoglobulin is produced locally in the gland after IgA expressing B-lymphocytes that had contact with antigen home to secretory epithelia [513]. Based on this fact we expected differences in the IgA titer in milk between supramammary and parotid lymph nodes areas in the vaccinated groups; however, there were no differences in GapC/B titers in intradermal vaccination at both sites. This may be due to several reasons. For example, vaccination near the supramammary and parotid lymph nodes might induce the production of IgA with equal magnitude in spite of the fact that the supramammary lymph node is the draining lymph node near the mammary gland. On the other hand, local production might be high but secretion into milk and serum might be limited or not efficient and there might be local differences in efficient presentation of antigen to immune effector cells. It is very important to understand the mechanism involved in the local IgA production in the bovine mammary gland, especially in how IgA secreting B-cells home to the lamina propria of the mammary gland after contact with antigen. Also receptors and mediators involved in the signalling pathways need to be known to design better immunization strategy for increasing local production of IgA. Moreover, receptors and mediators involved in the process of IgA secretion from local sites into milk need to be clearly defined to target them for enhancement of local production of IgA in milk.



## **4.4. Monitoring Duration of Immune Responses in Cows.**

### **4.4.1. Introduction**

The physiology of a mammary gland is continuously changing throughout the animal's reproduction cycle to meet the requirement for the nourishment of the newborn. The resistance and susceptibility to intramammary infection also varies at different stages of the reproduction cycle. In dairy cows the mammary gland is susceptible to intramammary infections during early non-lactating and during periparturient periods whereas in the mid to late lactation period the mammary gland is relatively better protected. The number of neutrophils and the concentration of immunoglobulins are high both during the early non lactating period and during prepartum; however the udder is not protected against bacterial infections [515]. The reasons for increased susceptibility to mastitis during these periods are not clearly understood, two factors that might contribute are cessation of hygienic milking time practices which greatly help in avoiding colonization of the teat end and udder skin during early lactation, and the anti-inflammatory effects of hormones that induce parturition (calving) such as cortisol. Detailed understanding of the profile of intramammary immunity over the reproduction cycle is of paramount importance for better design of protective vaccines against intramammary infections. Antigen is only one component of the vaccine. Adjuvants, which increase the magnitude of the immune responses, play a critical role in vaccine efficacy. Novel adjuvants that rapidly induce Th-1 responses as well as opsonizing antibodies production by B cells of immunized cows are also very important. Most veterinary vaccines are still formulated with oil-based adjuvant and are delivered in a fashion which stimulates humoral, but not local immunity. Therefore, it is necessary to find novel adjuvants that can increase the magnitude of the immune response. CpG ODN (cytosine-phosphate-guanosine oligodeoxynucleotides) and PCPP (Poly [di (carboxylatophenoxy) phosphazene) are some synthetic molecules that have potential to be used as adjuvants. CpG oligonucleotides are small DNA molecules that belong to the family of pathogen-associated molecular patterns (PAMP). Unmethylated CpG dinucleotide in a specific base sequence conferred immunostimulatory activity to bacterial DNA (Krieg et al., 1995). Synthetic oligodeoxynucleotide (ODNs) containing this same sequence also had immunostimulatory

activity (Krieg et al., 1995). The CpG motif binds to the Toll-like receptor 9 (TLR9) molecules and upon recognition, cell-signalling pathways are induced resulting in pro-inflammatory responses and predominantly Th-1-like immune responses (reviewed in [458, 492]. Poly [di(carboxylatophenoxy) phosphazene] (PCPP) is a water soluble synthetic biodegradable products that has been proven to be immunostimulatory in mice[516]. The PCPP has been shown to have adjuvant activity with many viral and bacterial antigens in mice [516-518]. It has been shown to increase the IgM, IgG, IgG1 and IgG2a titers against influenza antigens and IgG against tetanus toxoid in mice [516, 519, 520]. Recently, it has been shown that the polyphosphazene-CpG ODN combination of adjuvants enhances immune responses in mice more effectively than either of the individual adjuvants [521]. These authors also showed that PCPP induced innate cytokine production, which might indicate a mechanism by which polyphosphazenes induce their adjuvant role. In another study, polyphosphazene polymer 6 was shown to have similar potent immune-adjuvant effect when formulated with CpG-ODN in mice [522]. Its adjuvant activity has not been tested in large animals although it has been reported to be a potent adjuvant in sheep when used at twice the dose for mice (Gorge Mutwiri personal communication).

This animal trial was designed to test vaccine formulations containing the adjuvant PCPP, the immunomodulator CpG, CpG + PCPP as well as VSA3 (oil in water) and the duration of the immune response to the GapC/B antigen. Forty cows (Five groups of eight) (Table 3.8.1) were selected from a dairy herd based on their clinical history and low basal levels of antibody against the GapB and GapC proteins. Blood and milk samples were taken before the vaccination (Day 0) and at days 21, 42, 60, 90, 120,150, 180, 210, 240, 270, 300, 330 and 360, which cover one lactation cycle. The antibody levels (total IgG, IgG1, IgG2, and IgA) in milk and serum were measured by ELISA. When the antibody levels decreased 20 fold as compared to the levels at day 42, animals were boosted. This monitoring of humoral immune responses was conducted throughout the lactation cycle to establish if there was a consistent correlation between antibody titres from serum and mammary secretions.

#### **4.4.2. Humoral Immune Responses in Serum and Milk of Cows.**

The results from these experiment showed that in serum, except for day 120 ( $p < 0.05$ ), the total IgG titres on the rest of the sampling days were significantly higher than the titres on day 0 ( $p < 0.001$ ). The IgG1, IgG2 and IgA titres were significantly higher than the titres on day 0 on all the sampling days ( $p < 0.001$ ). In animals vaccinated with the GapC/B chimera and CpG, PCPP or a combination of CpG + PCPP (Groups 3, 4, and 5 respectively) as adjuvants (Table 3.8.1), the serum levels of IgG, IgG1 and IgG2 to GapB, GapC, or GapC/B did not significantly increase with respect to pre-immunization levels. These results showed that the best formulation to confer a durable immune response to GapC/B in dairy cattle contains 30% VSA3 as adjuvant and the duration of humoral immune responses using GapC/B as antigen is approximately four months. In this experiment since all animals are not at the same stage of lactation (calving was not synchronised) the starting and ending of our immune response monitoring vary from individual animal to animal. Some of our animals dried off while others were still in lactation. This situation created a problem for accurate statistical analysis of intramammary immune response trend over the longitudinal period of one lactation cycle.

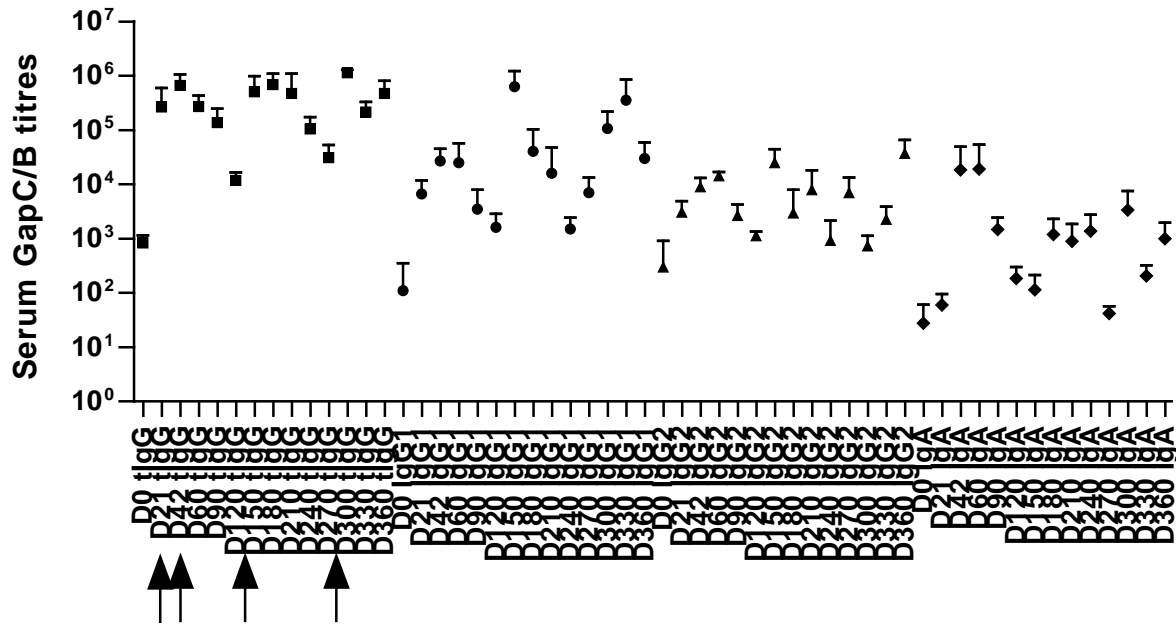


Figure 4.4.2.1. Duration of immune responses (Serum titers).

The mean logarithm (+ S.D.) of serum titers from animals vaccinated with the GapC/B chimera formulated in VSA3 (Group 2) and obtained at days 0, 21, 42, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 are shown. Animals were vaccinated on days 0, 21, 134 and 270 shown by arrows. Except for day 120 ( $p < 0.05$ ), the total IgG titers on the rest of the sampling days were significantly different than the titers on day 0 ( $p < 0.001$ ). The IgG1, IgG2 and IgA titers were significantly different from day 0 on all the sampling days ( $p < 0.001$ ). The antibody type and the sample days are indicated on the X axis. The serum levels at day 120 were below the threshold (20 fold the serum levels of day 42) and the animals received a boost on day 134 (14 days after day 120). The rest of the groups showed low serum levels at all the times tested. The arrows indicate the vaccination days.

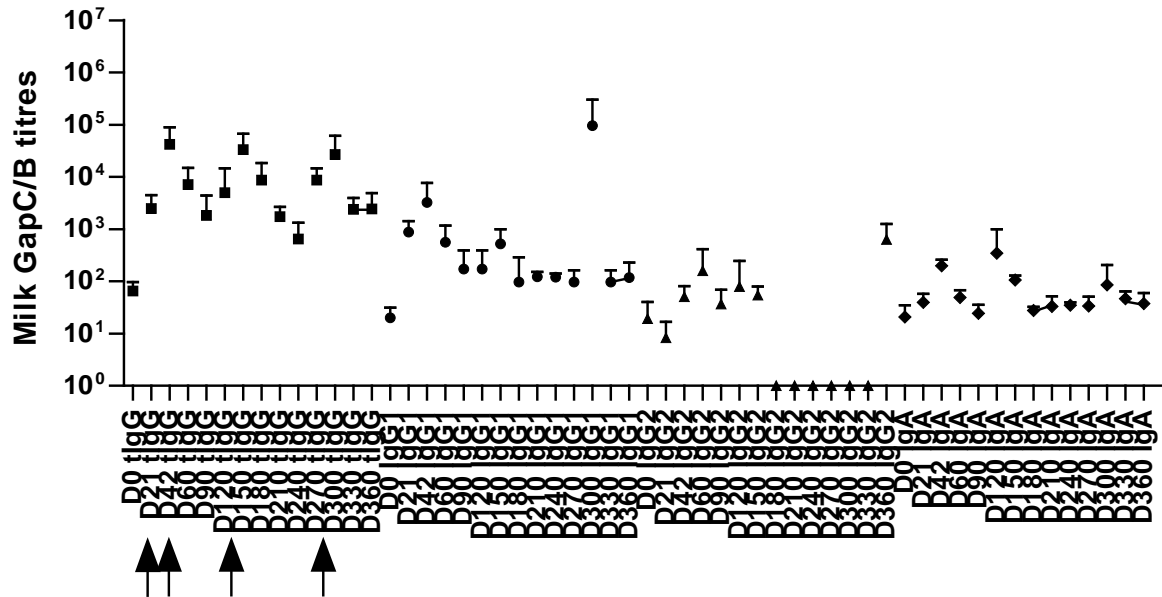


Figure 4.4.2.2. Duration of immune responses (Milk titers).

The mean logarithm (+S.D.) of milk titres from animals vaccinated with the GapC/B chimera formulated in VSA3 (Group 2) and obtained at days 0, 21, 42, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 are shown. Animals were vaccinated on days 0, 21, 134 and 270 shown by arrows. The antibody type and the sample days are indicated on the X axis. The milk titer levels at day 120 were below the threshold (20 fold the milk titer levels of day 42) and the animals received a boost on day 134 (14 days after day 120). The rest of the groups showed low milk titer levels at all the times tested. The arrows indicate the vaccination days.

#### 4.4.3. Discussion

These results showed that in serum except for day 120 ( $p < 0.05$ ), the total IgG titers on the rest of the sampling days were significantly higher than the titers on day 0 ( $p < 0.001$ ). The IgG1, IgG2 and IgA titers were significantly higher than the titers on day 0 on all the sampling days ( $p < 0.001$ ). The best formulation that confers a durable humoral immune response to GapC/B in dairy cattle is that containing 30% VSA as an adjuvant. This experiment is not sufficient to provide conclusive answers for the profile of immune responses throughout one lactation period using GapC/B as an antigen because we didn't monitor the cellular immune response. However, since the IgG2 titers can be an indication of cellular responses, by implication we expected the existence of cellular immune responses against GapC/B. Results from this experiment also showed that the duration of humoral immune response is approximately four months. The serum levels of GapB, GapC, or GapC/B did not significantly increase with respect to pre-immunization levels in animals vaccinated with CpG, PCPP or a combination of CpG + PCPP (Groups 3, 4, and 5, respectively) (Table 3.8.1). This might be due to interference between GapC/B and CpG. Polyphosphazines are known to enhance immune responses in mice, however their effect in large animals requires further evaluation.

## **4.5. Role of GapC in the Virulence of Bovine *Staphylococcus aureus* Mastitis.**

### **4.5.1. Introduction.**

*S. aureus* produces a wide array of cell surface and extracellular proteins involved in virulence. Expression of these virulence factors is tightly controlled by numerous regulatory loci, including *agr*, *sar*, *sigB*, *sae*, and *arl*, as well as by a number of proteins with homology to SarA. The *sar* locus regulates the expression of the *agr* locus which encodes an elaborate quorum-sensing system that was hypothesized to differentially regulate the expression of cell wall associated proteins and secreted exoproteins in response to the density of bacterial populations [208]. Amongst the cell-wall associated proteins of mastitis-isolates of *S. aureus* are the GapB and GapC proteins with homology to GAPDH [75]. Based on evidence collected which indicated that GAPDH is involved in other non-glycolytic functions, we hypothesized that staphylococcal GAPDH by the virtue of its surface localization may function as an adhesin for *S. aureus* colonization of host tissue surfaces including the bovine mammary gland. To evaluate this hypothesis, *in vitro* adhesion and invasion assays of *S. aureus* RN6390 and its isogenic *gapC* mutant H330 into MAC- T cells were conducted.

### **4.5.2. *In vitro* Adhesion and Invasion Assays of *Staphylococcus aureus* Strain RN6390 and its Isogenic GapC Mutant Strain H330 into Mammary Epithelial Cell Line (MAC-T).**

Results from *in vitro* adhesion and invasion assays on MAC- T cells, from 8 different experiments, each in triplicate, showed that the number of RN6390 bacteria that adhered (attached) to MAC-T cells at 37 °C (Fig. 4.5.2.1) and 4 °C (Fig. 4.5.2.2) was significantly ( $p<0.05$ ) higher than that of the isogenic GapC mutant strain H330. Similarly, the number of RN6390 invaded (internalized) into MAC-T cells at 37 °C and 4 °C was significantly ( $p<0.01$ ) higher than that of the isogenic GapC mutant strain H330. This suggests the involvement of GapC as a *S. aureus* cell-surface receptor for attachment to bovine mammary epithelial cells. The wild type strain RN6390 also killed MAC-T cells and was released into the supernatant 2, 3 and 4 hs after incubation in fresh media without antibiotics while the GapC mutant strain did not kill MAC-T cells since no viable counts were obtained after 2, 3 and 4 hs of incubation in fresh media without antibiotics (Fig. 4.5.2.3 and Fig. 4.5.2.4). Although viable, there was no apparent

intracellular growth of the GapC mutant and this might have contributed to the lack of cell lysis (Fig. 4.5.2.3). To determine whether attachment and internalization requires metabolically active cells, the adhesion and invasion assays were conducted at 4 °C and the result showed that the number of RN6390 and H330 cells that attached and/or internalized at 4 °C were significantly different and comparable to the numbers at 37 °C (Fig. 4.5.2.2). Although the numbers of bacteria of the GapC mutant strain H330 that attach and/or internalize into MAC-T cells were significantly less than the number of wild type bacteria the absence of GapC did not prevent the attachment and internalization. This could be due to the presence of several other molecules on the surface of *S. aureus* that are also known to serve as adhesins such as fibronectin binding proteins A and B [69, 70], fibrinogen binding proteins (Clumping factor A and B) [72, 73, 523], collagen binding protein [71], vitronectin binding protein [66, 524], elastin binding protein [74], extracellular adherence protein (Eap) [525] and many other surface components (Table 1.2.1.1). It has been shown that in other *S. aureus* strains deletion of the extracellular adherence protein [167, 526] did not affect binding of the mutant strain to host matrix components. These authors also suggested that the absence of a significant effect might be due to the presence of other adherence proteins such as fibronectin binding proteins, fibrinogen binding proteins and many other adhesins on the surface of *S. aureus*. In another similar study deletion of extracellular adherence proteins decreased internalization of *S. aureus* into human fibroblast and epithelial cells [68] and complementation of extracellular adherence protein restored internalization of the mutant strain into these cells [68]. In another study deletion of fibronectin binding proteins in *S. aureus* strain 8325-4 [527] reduced the adherence of the isogenic mutant strain to MAC-T cells by 40% and internalization by more than 95% [132]. Similarly, Brouillette et al. [528] reported that *S. aureus* strain DU5883 lacking fibronectin binding protein showed 30% reduction in its ability to attach to MAC-T cells as compared to its isogenic wild type strain 8325-4. More importantly these authors showed that the presence of fibronectin binding proteins in *S. aureus* strain 8325-4 increased the colonization of the lactating mammary gland in the mouse model of mastitis as compared to mutant strain DU5883 under *in vivo* conditions. Taken all these findings together it is possible to conclude that colonization of host tissue surfaces including bovine mammary gland by *S. aureus* is achieved by multiple cell surface adhesins and inactivation of one or some of them would not abrogate bacterial attachment to host tissue surfaces because of compensatory effects by others.



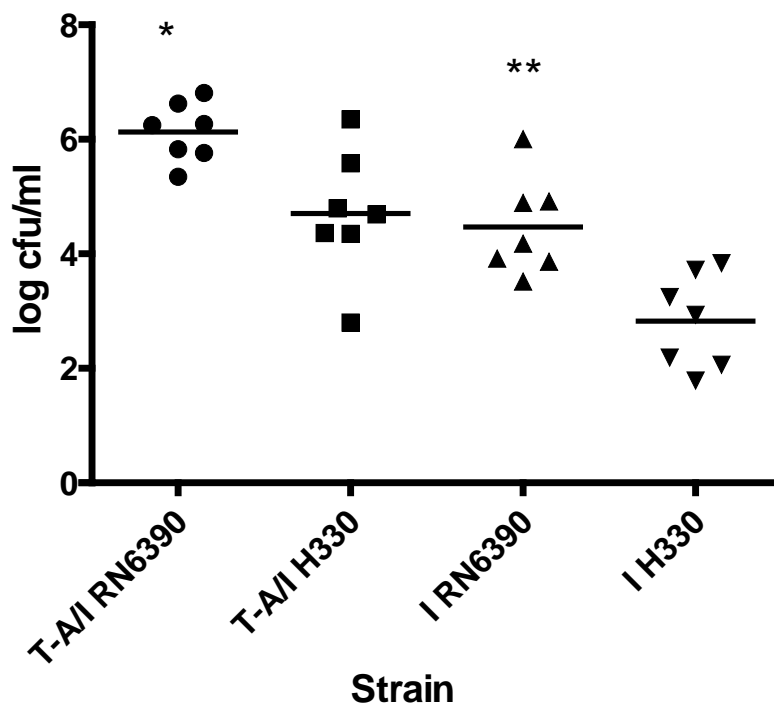


Figure 4.5.2.1. Number of RN6390 bacteria and its isogenic GapC mutant strain H330 attached and internalized into MAC- T cells at 37 °C.

The number of attached and/or intracellular bacteria ( $\pm$ S.D.) of wild type strain RN6390 at 37 °C was significantly higher than that of isogenic GapC mutant strain. T-A/I: Total number of attached and internalized bacteria; I: Internalized bacteria. The bars refer to mean of the total counts. The symbols above the bars indicate statistical differences between the groups (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ).

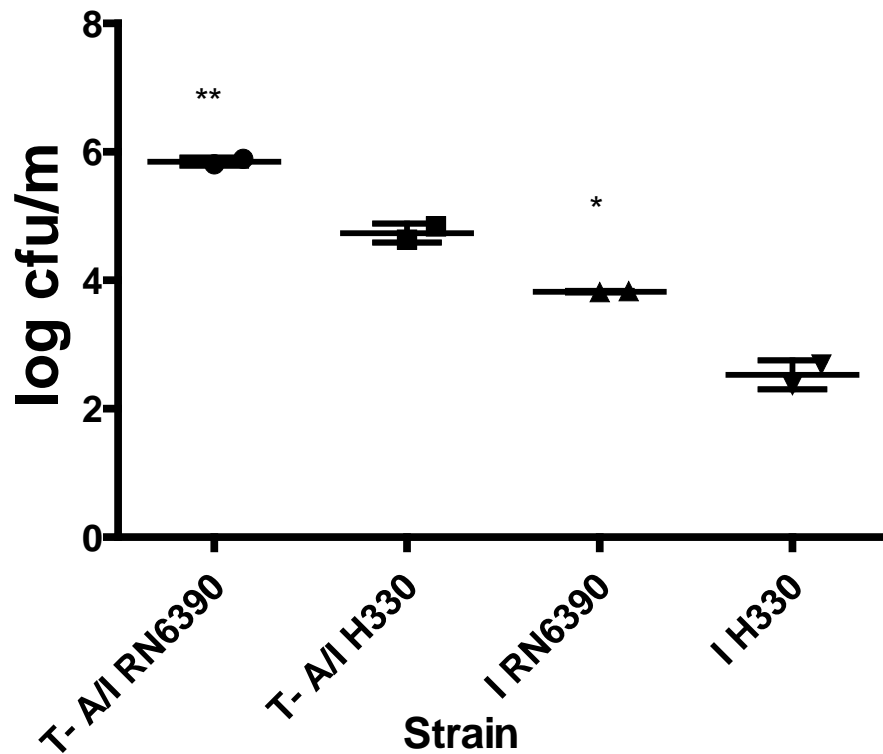


Figure 4.5.2.2. Number of RN6390 bacteria and its isogenic GapC mutant strain H330 attached and internalized into MAC- T cells at 4 °C.

The numbers of attached and/or internalized bacteria of wild type strain RN6390 at 4 °C was significantly higher than that of isogenic GapC mutant strain. T-A/I = Total number of attached and intracellular bacteria. The bar refers to mean of total counts. The symbols above the bars indicate statistical differences between the groups (\*=  $p < 0.05$ ; \*\*=  $p < 0.01$ ).

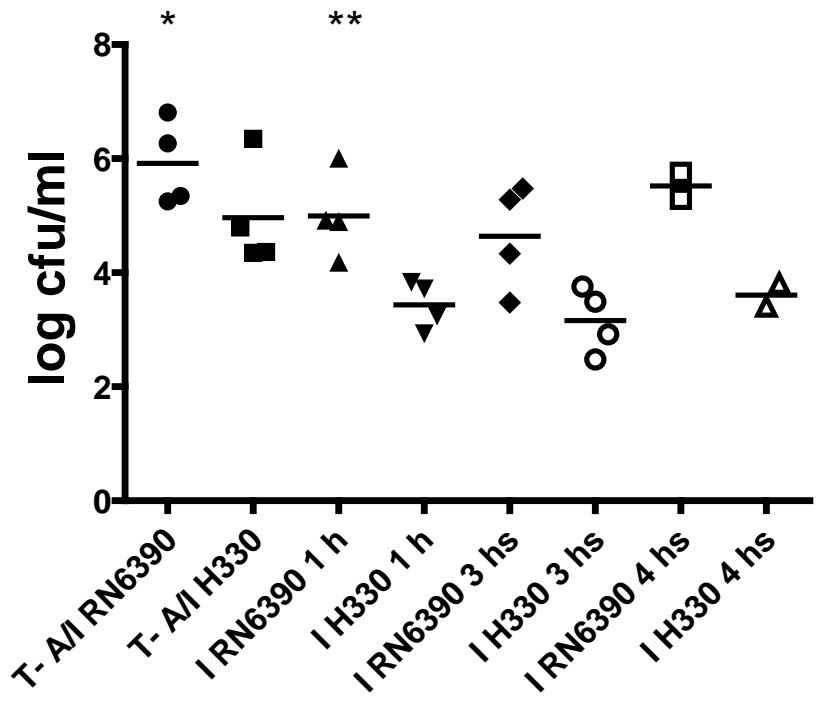


Figure 4.5.2.3. Number of intracellular bacteria recovered at different time points.

The number of intracellular bacteria does not significantly change over time. T-A/I = Total number of attached and intracellular bacteria, I= Intracellular. The bars refer to the mean of total counts.

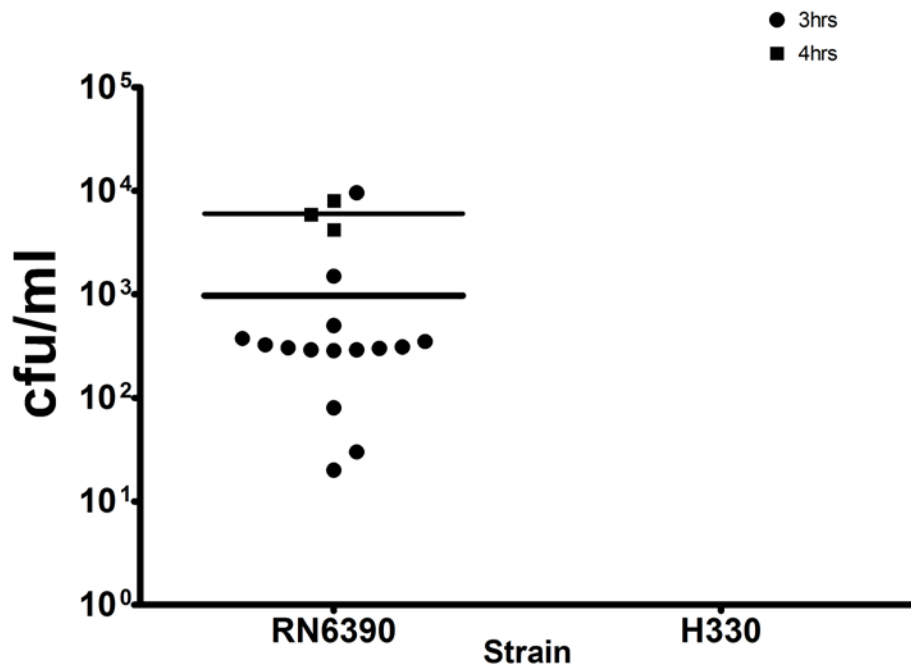


Figure 4.5.2.4. Number of intracellular bacteria released into supernatants after incubation in fresh media without antibiotics.

The thick bar indicates the mean of bacterial counts after 3hs while the thin bar indicates the mean of the bacterial count after 4hs of incubation. The mutant strain H330 was recovered from the supernatant which indicates that it did not kill MAC- T cells but stay in the intracellular area.

#### 4.5.3. Pilot Challenge Experiment Using the *Staphylococcus aureus* Strains RN6390 and its Isogenic GapC Mutant H330 on Ovine Mammary Gland

The ability of the GapC mutant *S. aureus* strain H330 to infect MAC-T cells has been determined *in vitro*. The results from this experiment showed that the adhesion and invasion ability of H330 into MAC-T cells was significantly lower than that of the isogenic wild type strain RN6390 suggesting that in an *in vitro* model the GapC mutant would be less pathogenic. To confirm this hypothesis we conducted a challenge trial. Ovine mammary glands were infected with RN6390 and H330 and we monitored the responses over 4 days. Because of very high expenses that we could not afford to do this experiment in cows, we decided to conduct it in ovine mammary glands. We have conducted pilot challenge experiments to determine optimum challenge dose for RN6390 and H330 in the ovine mammary gland. For the pilot trial we screened 6 Suffolk breed dairy sheep in the first week of lactation and selected 3 animals based on SCC and milk bacteriological culture results (Table 4.5.3.1). These animals (72M, 81P and

20P) were challenged with high ( $10^6$ ), medium ( $10^4$ ) and low ( $10^2$ ) CFU/ml doses of *S. aureus* strain RN6390 into the right side udder halves and H330 into left side udder halves respectively in a total volume of 1 ml.

Table 4.5.3.1 Pre-screening of animals for intramammary infection for pilot challenge trial

Anl. ID	Udder	Bacterial count and colony description	SCC	Remarks
20P	R	White transparent colony, 1 CFU/200 $\mu$ l Gram-positive rod.	$1.76 \times 10^8$	Selected
	L	Small size colony, 1 CFU/200 $\mu$ l, Gram-negative cocci.	$1.95 \times 10^8$	
72M	R	No growth.	$1.95 \times 10^7$	selected
	L	Small size colony, Gram negative cocci, 2 CFU/200 $\mu$ l	$2.27 \times 10^7$	
81P	R	No growth	$1.24 \times 10^8$	Selected
	L	Small size colony, about 5 CFU/200 $\mu$ l, Gram-negative cocci	$1.36 \times 10^8$	
154M	R	White circular colony, 2 CFU/200 $\mu$ l, Gram-positive cocci, staphylococci and small grayish colony of about 3 CFU/200 $\mu$ l, Gram-negative cocci.	$5.62 \times 10^7$	Not selected
	L	Similar colonies as above, staphylococci 1 CFU/200 $\mu$ l, Gram negative cocci 2 CFU/200 $\mu$ l.	$1 \times 10^8$	
194M	R	Small grayish, Gram-negative colonies ca. 20 CFU/200 $\mu$ l and 2 CFU/200 $\mu$ l of staphylococci.	$7.2 \times 10^7$	Not selected
	L	3 CFU/200 $\mu$ l of Gram-negative cocci, 1 CFU/200 $\mu$ l of staphylococci,	$6.95 \times 10^7$	
238P	R	3 CFU/200 $\mu$ l of staphylococci	$1.59 \times 10^8$	Not selected
	L	2 CFU/200 $\mu$ l of Gram-negative cocci and 1 CFU/200 $\mu$ l of staphylococci.	$1.83 \times 10^8$	

Abbreviations: L= Left side udder, R= Right side udder, Anl.= Animal, Anl. ID = animal identification number

Table 4.5.3.2. Pre-screening of animals for intramammary infection for *in vivo* challenge trial

Anl. ID	Udder	Milk culture bacterial count	SCC	Body temp. in °C	Remark
156	R	No growth	4.27 X 10 <sup>6</sup>	39.8	(Group 1)
	L	“	3.67 X10 <sup>6</sup>		
157	R	“	7.62 X 10 <sup>7</sup>	39.9	
	L	“	6.79 X 10 <sup>6</sup>		
158	R	“	2.83 X 10 <sup>8</sup>	39.7	
	L	“	2.46 X 10 <sup>6</sup>		
159	R	“	2.02 X 10 <sup>6</sup>	39.5	
	L	“	1.60 X 10 <sup>7</sup>		
160	R	“	2.07 X 10 <sup>8</sup>	39.4	
	L	“	5.52 X 10 <sup>7</sup>		
161	R	“	2.69 X 10 <sup>6</sup>	39.7	(Group 2)
	L	“	3.13 X 10 <sup>6</sup>		
162	R	“	3.65 X10 <sup>7</sup>	39.8	
	L	“	7.27 X 10 <sup>6</sup>		
163	R	5 CFU/ml Gram negative rod	2.87 X 10 <sup>6</sup>	39.6	
	L	7 CFU/ml Gram negative rods	2.133 X 10 <sup>6</sup>		
164	R	No growth	3.24 X 10 <sup>6</sup>	39.3	
	L	“	8 X 10 <sup>8</sup>		
165	R	3 CFU/ml Gram negative cocci	1.29 X 10 <sup>8</sup>	39.1	
	L	2 CFU/ml Gram negative cocci	2.47 X 10 <sup>6</sup>		
3R	R	2 CFU/ml Gram negative cocobacilli	6.67 X 10 <sup>6</sup>	39.6	Not selected
	L	50 CFU/ml Gram negative coccobacilli	5.66 X10 <sup>7</sup>		
26T	R	No growth	1.35 X 10 <sup>7</sup>	39.2	Not selected
	L	3 CFU/ml Gram positive cocci	2.95 X 10 <sup>6</sup>		
116P	R	2 CFU/ml Staphylococci	4.29 X 10 <sup>6</sup>	39.3	Not selected
	L	59 CFU/ml Staphylococci	1.42 X 10 <sup>7</sup>		
20T	R	No growth	3.85 X 10 <sup>6</sup>	39.4	Not selected
	L	3 CFU/ml Gram positive cocci	1.64 X 10 <sup>7</sup>		
61S	R	19 CFU/ml Gram positive cocci	1.48 X 10 <sup>8</sup>	39.0	Not selected
	L	3 CFU/ml Gram positive cocci	1.37 X 10 <sup>6</sup>		

Abbreviations: L= Left side udder, R= Right side udder, Anl.= Animal, Anl. ID = animal identification number

Table 4.5.3.3. Summary of pre- and post-challenge bacterial and somatic cell counts in both trials.

Anl. ID	Udder	Prechallenge bacterial count	Prechallenge Somatic cell count/ml	Challenge strain and dose/ml	Post challenge bacterial count/ml		Post challenge somatic cell count/ml	
					24hrs	48hrs	24hrs	48hrs
20P	R	No growth	1.07 X 10 <sup>8</sup>	RN6390 4.30 X 10 <sup>2</sup>	6.83 X 10 <sup>6</sup>	8.3 X 10 <sup>7</sup>	1.14X 10 <sup>8</sup>	2.87X 10 <sup>8</sup>
	L	2 CFU/200µl of Gram negative cocci	1.27 X 10 <sup>8</sup>	H330: 3.90 X 10 <sup>2</sup>	6.1 X 10 <sup>5</sup>	LDL	6.76 X 10 <sup>6</sup>	1.86 X 10 <sup>6</sup>
72M	R	No growth	1.67 X 10 <sup>7</sup>	RN6390: 4.5 X 10 <sup>6</sup>	5.1X10 <sup>8</sup>	6.6 X10 <sup>7</sup>	2.66 X 10 <sup>8</sup>	5.76 X 10 <sup>8</sup>
	L	No growth	2.54 X 10 <sup>7</sup>	H330: 3.7 X 10 <sup>6</sup>	166	LDL	7.27 X 10 <sup>6</sup>	4.10 X10 <sup>6</sup>
81P	R	No growth	4.31 X 10 <sup>7</sup>	RN6390: 2.0 X 10 <sup>4</sup>	2.6 X 10 <sup>8</sup>	1.1 X 10 <sup>8</sup>	9.79X10 <sup>8</sup>	9.12 X 10 <sup>8</sup>
	L	1 CFU/200µl Gram negative cocci	5.50 X 10 <sup>7</sup>	2.15 X 10 <sup>4</sup>	LDL	LDL	1.96 X 10 <sup>7</sup>	5.6 X10 <sup>6</sup>
156	R	No growth	4.27 X 10 <sup>6</sup>	H330: 4.16 x 10 <sup>6</sup>	“	9	4.59 X 10 <sup>6</sup>	1.02X10 <sup>6</sup>
	L	“	3.67 X10 <sup>6</sup>	“	“	LDL	4.51 X 10 <sup>6</sup>	2.95X10 <sup>6</sup>
157	R	“	7.62X 10 <sup>7</sup>	“	“	“	1.36 X 10 <sup>7</sup>	8.12X10 <sup>6</sup>
	L	“	6.79X10 <sup>6</sup>	“	“	“	3 X10 <sup>6</sup>	2.89X10 <sup>6</sup>
158	R	“	2.83 X 10 <sup>8</sup>	“	“	9X10 <sup>3</sup>	8.74 X 10 <sup>7</sup>	1.7 X10 <sup>6</sup>
	L	“	2.46 X10 <sup>6</sup>	“	“	LDL	4X10 <sup>6</sup>	6.66X10 <sup>6</sup>
159	R	“	2.02 X 10 <sup>6</sup>	“	“	“	3.05 X10 <sup>6</sup>	2.31X10 <sup>6</sup>
	L	“	1.608 X10 <sup>7</sup>	“	113	“	1.83 X10 <sup>6</sup>	1.53X10 <sup>6</sup>
160	R	“	2.07 X10 <sup>8</sup>	“	LDL	“	2.51 X10 <sup>7</sup>	1.75X10 <sup>6</sup>
	L	“	5.52X 10 <sup>7</sup>	“	“	“	2.15X10 <sup>7</sup>	4.39X10 <sup>6</sup>
161	R	“	2.69 X 10 <sup>6</sup>	RN6390: 140	3.3X10 <sup>4</sup>	2X10 <sup>5</sup>	4.33 X10 <sup>7</sup>	3.82X10 <sup>8</sup>
	L	“	3.13 X10 <sup>6</sup>	“	1.6 X10 <sup>4</sup>	1X10 <sup>5</sup>	5.54 X 10 <sup>7</sup>	5.99X10 <sup>8</sup>
162	R	“	3.65 X10 <sup>7</sup>	“	2.16 X10 <sup>5</sup>	2.16X10 <sup>6</sup>	2.27 X10 <sup>7</sup>	2.21X10 <sup>8</sup>
	L	“	7.27 X 10 <sup>6</sup>	“	1.8X10 <sup>5</sup>	2.5 X10 <sup>6</sup>	5.19 X10 <sup>7</sup>	7.08X10 <sup>7</sup>
163	R	5 CFU/ml Gram negative rod	2.87 X10 <sup>6</sup>	“	1.8X10 <sup>4</sup>	9X10 <sup>5</sup>	7.25X10 <sup>7</sup>	7.8X10 <sup>7</sup>
	L	7 CFU/ml Gram negative rods	2.133 X10 <sup>6</sup>	“	2.16X10 <sup>3</sup>	1.6X10 <sup>5</sup>	9.11 X10 <sup>7</sup>	6.93X10 <sup>8</sup>
164	R	No growth	3.24 X10 <sup>6</sup>	“	2X10 <sup>5</sup>	8.3X10 <sup>5</sup>	4.35X10 <sup>7</sup>	6.6X10 <sup>8</sup>
	L	“	8 X10 <sup>5</sup>	“	5X10 <sup>4</sup>	2.8X10 <sup>5</sup>	1.13X10 <sup>8</sup>	6.83X10 <sup>7</sup>
165	R	“	1.29X10 <sup>8</sup>	“	1X10 <sup>5</sup>	7.1X10 <sup>5</sup>	2.05 X10 <sup>8</sup>	4.66X10 <sup>8</sup>
	L	“	2.47 X10 <sup>6</sup>	“	5X10 <sup>4</sup>	2.3 X10 <sup>6</sup>	2.66X10 <sup>7</sup>	1.75X10 <sup>8</sup>

Abbreviations: L= Left side udder, R= right side udder, LDL= less than detectable level

Table 4.5.3.4. Peripheral blood mononuclear cells (PBMCs) count and number of intracellular bacteria after 24 hs of intramammary challenge

Animal	PBMCs/ml	Number of intracellular bacteria	Temperature in °C
20P	1.26 X 10 <sup>7</sup>	340 CFU/ml	40.9
72M	9.11 X 10 <sup>6</sup>	1000 CFU/ml	39.9
81P	8.43 X 10 <sup>6</sup>	60 CFU/ml	39.3

The number of intracellular *S. aureus* recovered from bovine PBMCs is shown as well as the body temperature of the animals.

#### 4.5.3.1. Clinical Findings, Milk Bacteriological Culture Results and Somatic Cell Counts

All three animals in the pilot challenge trial were healthy and their feed intake was normal before challenge. After 24 hs of intramammary infusions all three animals were sick, some of the clinical signs included loss of appetite, weakness, depression and slightly increased body temperature to 40.9 °C, 39.9 °C and 39.3 °C for 20P, 72M and 81P respectively. The right side udder halves that received the wild type strain were swollen and bluish in color in all three animals which was an indication of gangrene development. Milk from all three right side udder halves of 20P, 72M and 81P animals had clots and was bloody in color. Milk from the left side udder halves that received H330 had no detectable changes in all animals. There were no clinically detectable changes in these udder halves. In this pilot experiment because of the limited number of animals, each of three animals received wild type bacteria into its right side udder and mutant into the left side udder. Because of that we were not sure whether the response was only from wild type bacteria or if the mutant strain also contributed to the systemic effects.

Milk bacteriological culture results showed that all three right side udder halves challenged with wild type strain RN6390 had high bacterial counts both at 24 hs and 48 hs post challenge whereas only two of left side udder halves (20P and 72M) had bacterial count of 10<sup>5</sup> and 10<sup>2</sup> CFU/ml respectively at 24 hs post challenge (Table 4.5.3.3). After 48 hs post challenge



there were no bacterial counts from the three left side udders challenged by the mutant strain whereas counts from right side udders challenged with wild type strain was high in all udders and increased by 2 logs in the 20P udder that received a low dose of  $10^2$  CFU/ml. The absence of growth from udders challenged with the mutant strain at 48 hs might be due to removal of the mutant strain by the defense system.

The somatic cell counts were relatively higher in all three animals even before challenge. However; their milk bacteriological culture results were negative except for 20P and 81P which have 2 and 1 CFU/ml of Gram negative cocci from the left side udder halves respectively (Table 4.5.3.3). It has been indicated that in ewes generally somatic cell counts are high during early and late lactation and also that the SCC from healthy udders of ewes are variable and could reach up to  $10^6$  cells /ml of milk. In addition to this, the older ewes usually have high somatic cell counts compared to younger ones. In this case all three ewes are older and this might contribute to high somatic cell counts from these animals. Although they have high somatic cell counts before challenge, the somatic cell counts from udder halves challenged by mutant strain H330 started dropping after 24 hs whereas counts from udder halves challenged with the wild type strain remained high throughout the follow up period of 48 hs (Table 4.5.3.3).

Peripheral blood mononuclear cells (PBMCs) were isolated and the presence of intercellular bacteria was analysed. Results from this experiment showed that peripheral blood mononuclear cells from all three animals were found to have significant numbers of intracellular bacteria which is an indication of bacterial uptake by phagocytic cells (Table 4.5.3.4).

**a. Udder halves challenged with  $10^6$  CFU/ml**



Right half (72R):  
Challenge strain wild type RN6390,  
observed findings: udder half was  
swollen and bluish in color and milk  
was bloody.

Left half (72L):  
Challenge strain GapC mutant H330,  
observed findings: no physically detectable  
changes in milk and udder half

**b. Udder halves challenged with  $10^4$  CFU/ml**



Right half (81R):  
Challenge strain RN6390,  
observed findings: udder half was  
swollen and bluish in color and  
milk was bloody.

Left half (81L):  
Challenge strain H330, observed  
findings: no physically detectable  
changes in milk and udder halves.

a. Udder halves challenged with  $10^2$  CFU/ml



Right half (20R):  
Challenge strain RN6390, observed findings: udder half was swollen and bluish in color and milk was bloody.



Left half (20L):  
Challenge strain H330, observed findings: No physically detectable changes in milk and udder half.

Figure 4.5.3.1a-c. Clinical udder examination results after 48 hs of intramammary challenge with RN6390 and its isogenic GapC mutant strain H330.

#### 4.5.3.2. Gross Pathological and Histopathological Findings

Gross pathological examination results of udder halves challenged with wild type strain RN6390 48 hs post challenge showed high accumulations of bloody exudates and the udder tissue was dark colored and swollen (Fig. 4.5.3.2a-c). The udder tissue challenged with the mutant strain had no grossly visible changes (Fig. 4.5.3.2a-c). The examination results of hematoxylin and eosin stained udder tissues challenged with the wild type strain showed massive infiltration of inflammatory cells, especially neutrophils (Fig. 4.5.3.3d-f). In udder tissue challenged with high dose of  $10^6$  CFU/ml of the wild type strain RN6390 there was necrosis and masses of necrotic debris seen whereas in the udder tissue which received medium and low doses of  $10^4$  and  $10^2$  CFU/ml respectively, there were large infiltrations of inflammatory cells with less necrosis (Fig. 4.5.3.3d-f). In udder tissue challenged with the mutant strain there were no visible histopathological changes (Fig. 4.5.3.3a-c). The examination results of Gram stained udder tissue challenged with the wild type strain revealed high number of

Gram positive cocci (Fig. 4.5.3.4a-c) whereas there were no stainable bacteria in udder tissue challenged with the mutant strain.

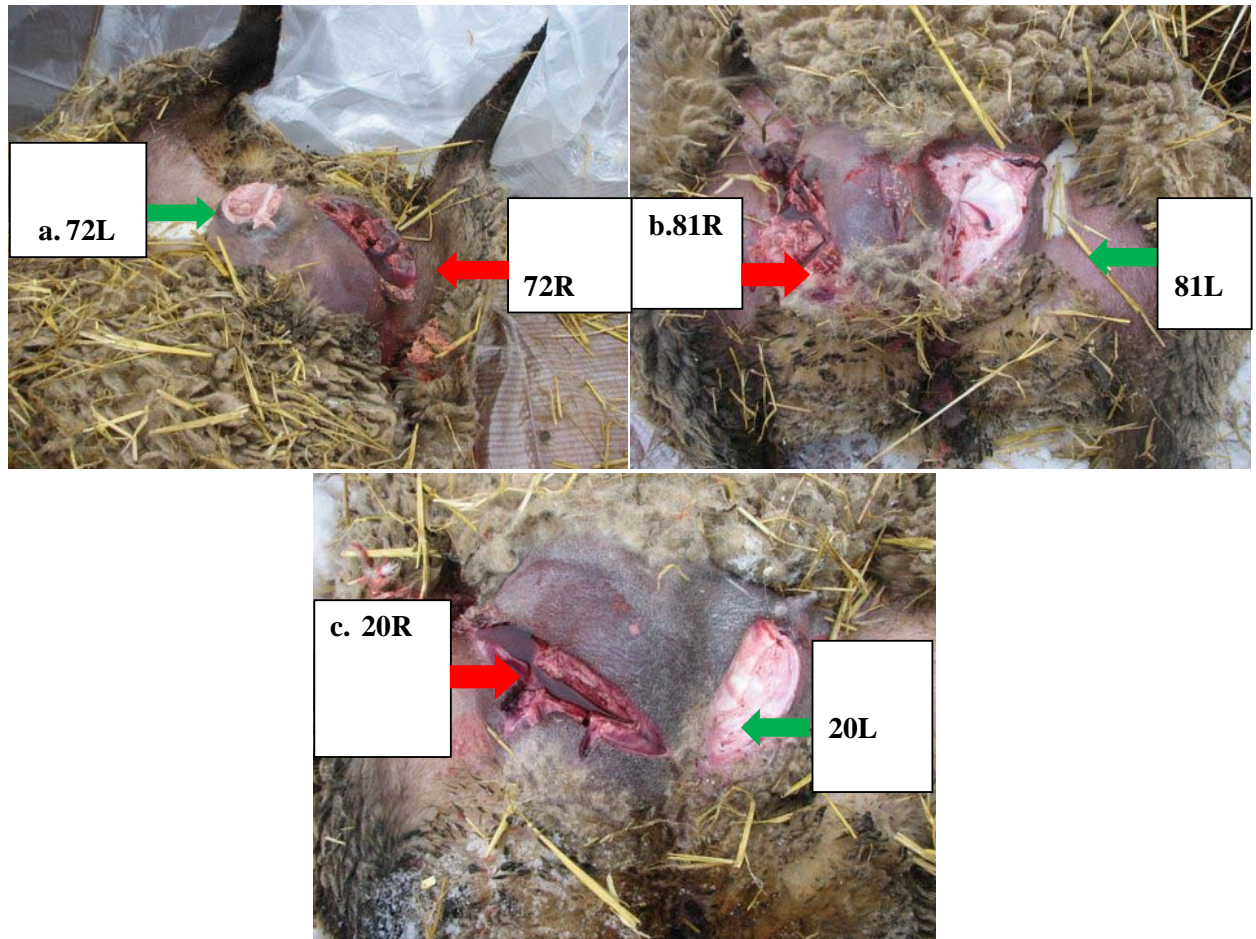


Figure 4.5.3.2a-c. Gross pathological changes in the right side udder halves that received wild type strain.

All udder halves challenged with wild type strain had high accumulation of bloody exudates. These udder halves were extremely swollen by high accumulation of bloody fluid as compared to the left udder halves that received the mutant strain.

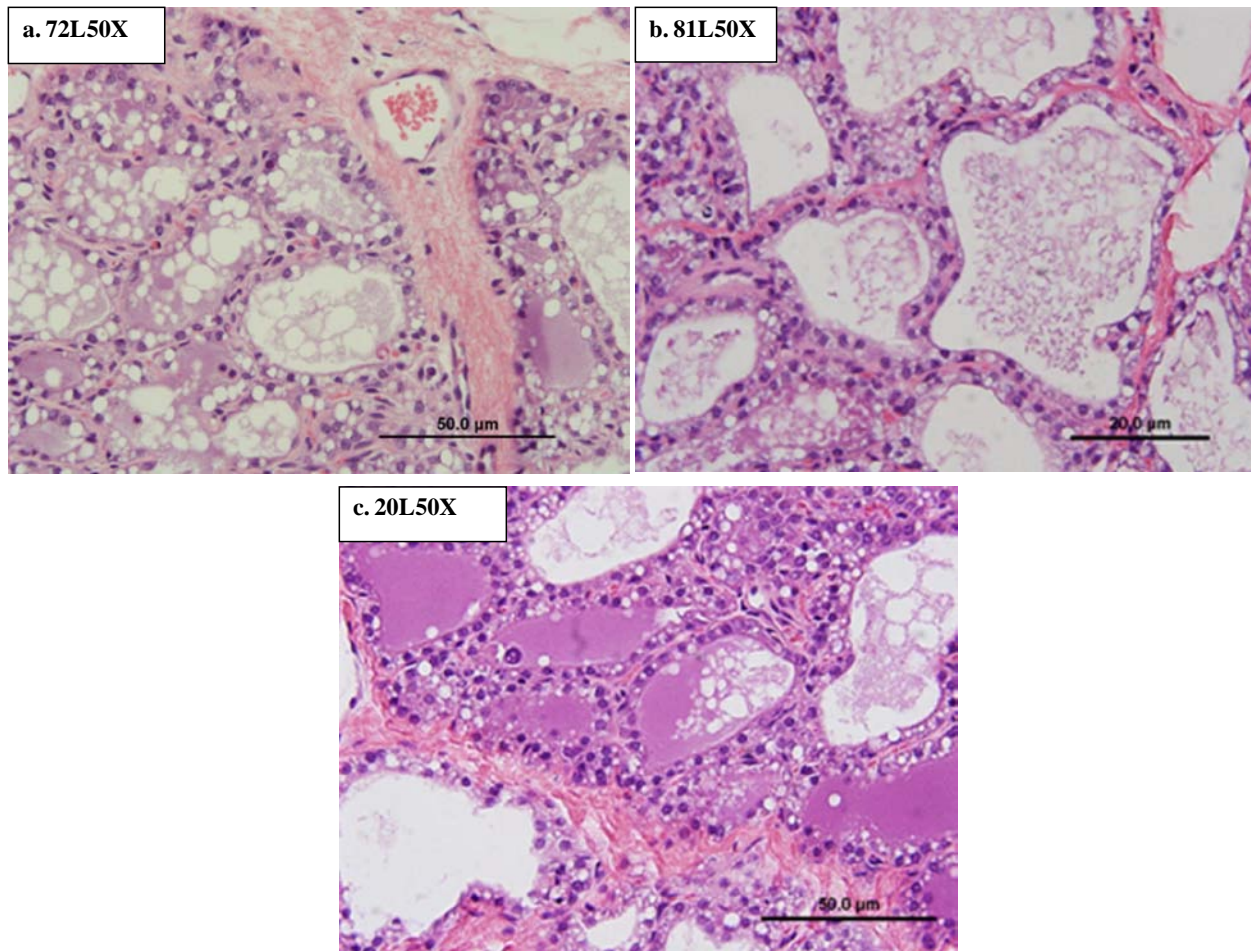


Figure 4.5.3.3a-c. Haematoxylin and eosin stained mammary gland tissue experimentally challenged with GapC mutant strain H330.

All three doses of the GapC mutant strain did not cause any change in the mammary gland tissue.

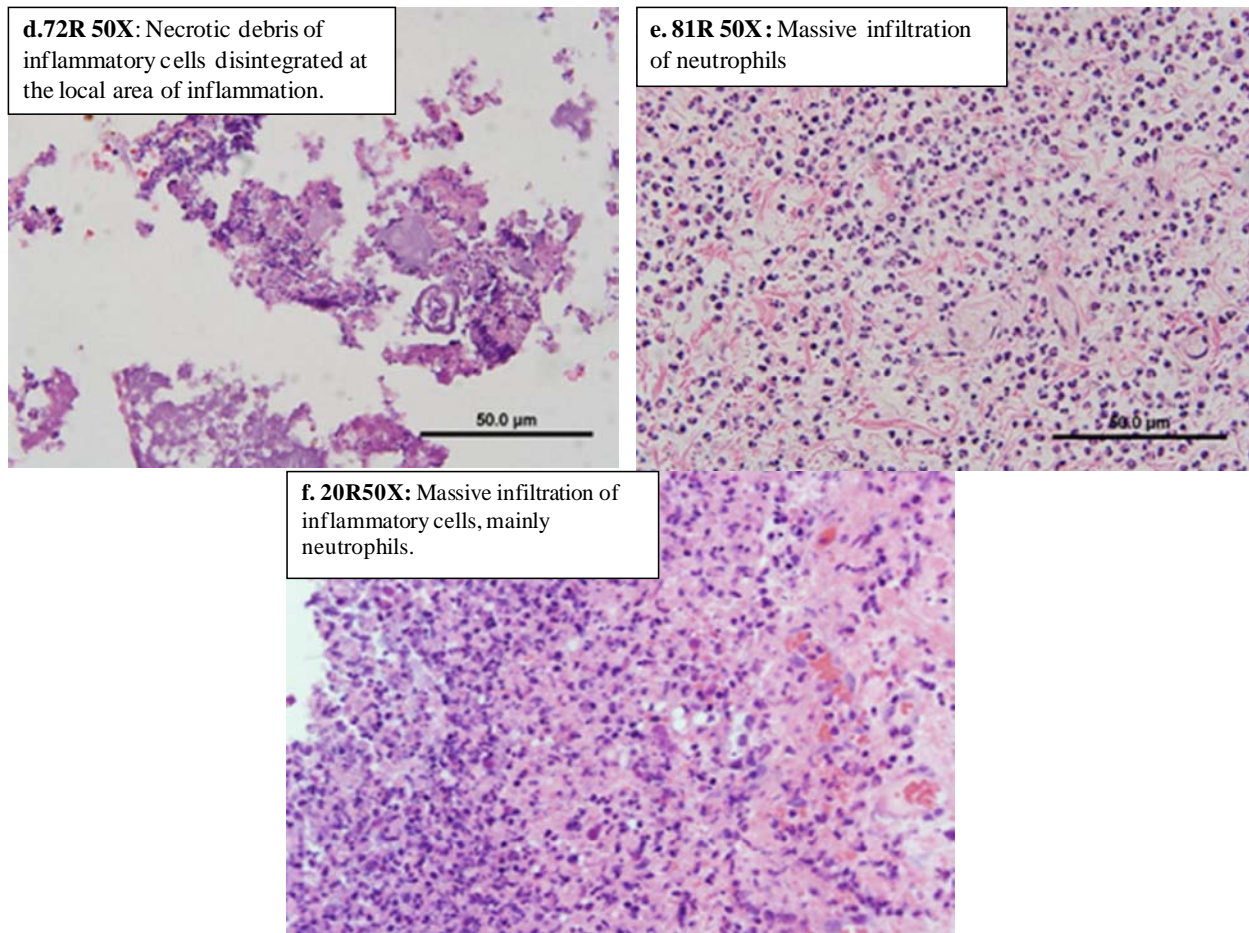


Figure 4.5.3.3d-f. Haematoxylin and eosin stained mammary gland tissue experimentally challenged by wild type *S. aureus* strain RN6390.

All three doses of wild type strain caused acute inflammation characterized by infiltration of inflammatory cells especially neutrophils.

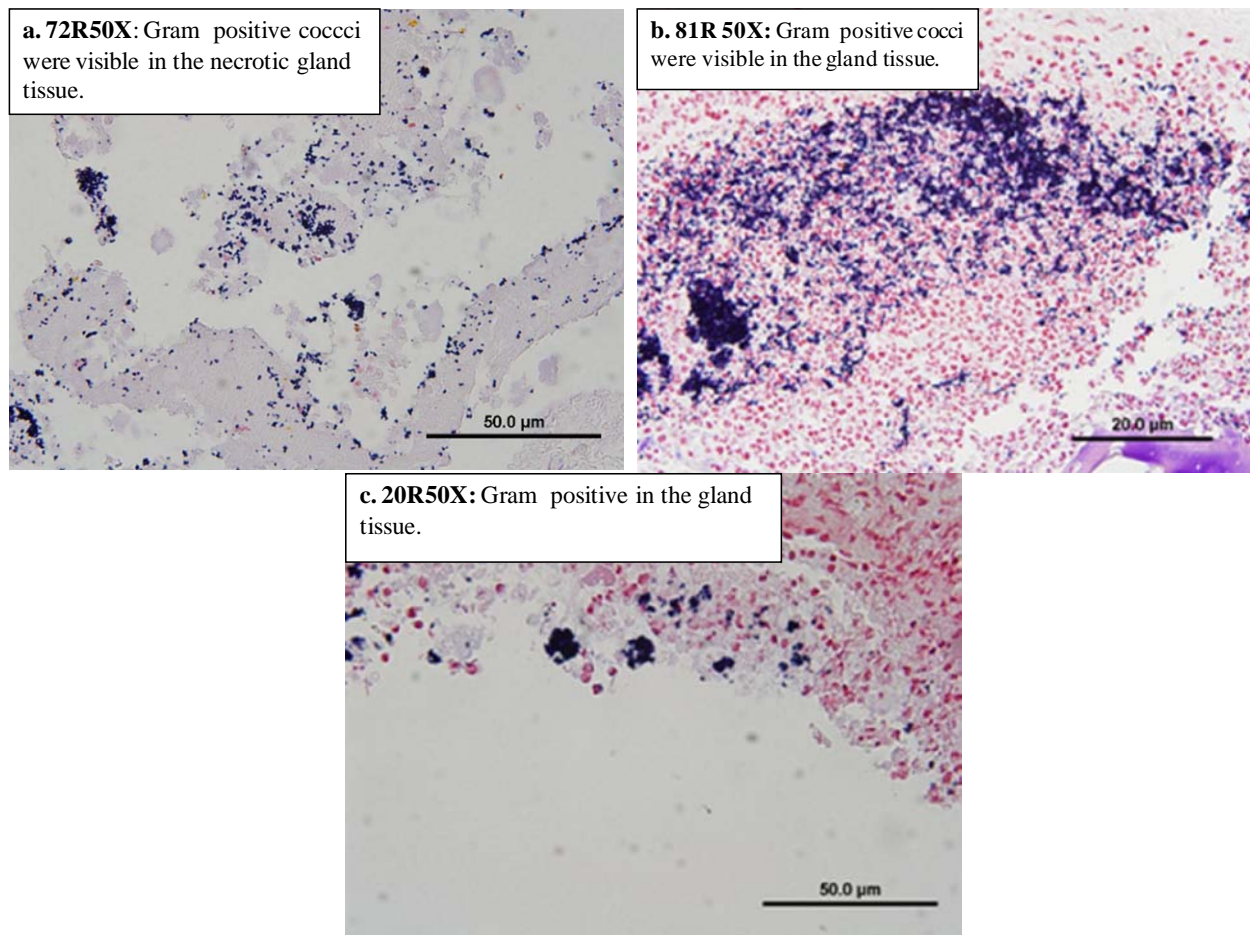


Figure 4.5.3.4a-c. Gram-stained mammary gland tissue from wild type strain RN6390 challenged udders.

Gram-positive cocci are visible in all udders challenged by wild type strain.

Conclusions from pilot challenge trial:

1. The wild type *S. aureus* strain RN6390 is highly pathogenic for Suffok ewes. The challenge doses of  $10^6$ ,  $10^4$  and  $10^2$  CFU/ml were very high and animals developed severe acute mastitis that led to lameness and gangrenous udders within 24 hs.
2. The GapC mutant strain H330 did not cause any detectable clinical or histopathological changes in the udder and milk.

Based on this result we decide to decrease the challenge dose for the wild type strain RN6390 to 50 CFU/udder.

#### **4.5.4. Mastitis Challenge Trial with the *Staphylococcus aureus* Strain RN6390 and its Isogenic GapC Mutant Strain H330 on Ovine Mammary Gland (second trial)**

After the preliminary challenge trial we decided to conduct the challenge trial with increased animal numbers to determine whether these findings are statistically significant or not. We also decided to decrease the challenge dose for the wild type strain to 50 CFU/udder while that of the GapC mutant strain was maintained at  $10^6$  CFU/udder. The challenge protocol for the second trial is presented in (Table 4.5.3.2). The second trial was comprised of 10 Suffolk breed dairy sheep in the first week of lactation in two groups of five sheep per group. All sheep in Group 1 were challenged with the GapC mutant *S. aureus* strain H330 whereas sheep in Group 2 were challenged with the isogenic wild type strain RN6390.

##### **4.5.4.1. Clinical Findings, Post-Challenge Milk Bacteriological Culture Results and Somatic Cell Counts**

As expected, clinical findings in wild type strain-infused sheep were not as acute as in the pilot study because the challenge dose was lower; however, after 48 hs all animals infused with the wild type strain developed clinical mastitis characterized by swollen udders with a hard consistency, slightly discolored milk with moderate milk clots, slightly increased body temperature although all animals were feeding and none appeared depressed. All animals infused with the mutant strain were healthy and there were no visible clinical signs of mastitis either locally in the mammary gland or systemically.

In general, milk bacteriological culture results were similar to the results seen in the pilot study in terms of bacterial recovery, i.e. only very few numbers of mutant bacteria were isolated from the right side udder halves of animals 159 and 158 and the left side udder half of animal 156 at 24 hs post challenge and none were isolated at 48 and 72 hs post challenge. All udder halves challenged with the wild type bacteria had high bacterial counts at 24 and 48 hs (Table 4.5.3.3) as well as at 72 hs (data not shown). The numbers of bacteria were slightly increased from 24 to 48 hs and the total numbers of bacteria were less than the number of bacteria counted at the same



time point for the pilot study, which may have been due to the low challenge dose (Table 4.5.3.3).

Similar to animals in the pilot study, pre-challenge somatic cell counts were also high in some udder halves of these animals but relatively better than that of pilot study since most udder halves had counts in the normal acceptable range. Post-challenge somatic cell counts were increased and remained high throughout the monitoring period of 72 hs in all animals challenged with the wild type strain RN6390 (Table 4.5.3.3). Similar to the pilot study, all udder halves challenged with the mutant strain had somatic cell counts that were slightly decreased, indicating that the mutant strain had no effect.

#### 4.5.4.2. Gross Pathological and Histopathological Findings

In animals challenged by wild type bacteria, a gross pathological examination after 72 hs revealed high accumulations of bloody exudates in all udder halves whereas in animals infused with the mutant strain H330 the udder halves looked normal and there were no grossly visible changes in the udder tissue.

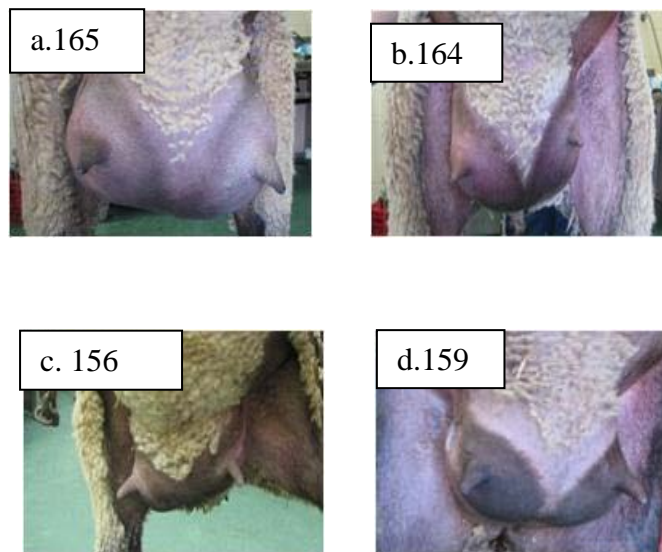


Figure 4.5.4.2. Gross pathological findings in udder halves challenged by wild type RN6390 (a and b) and mutant strain H330 (c and d).

Histopathological examination of udder tissues challenged with the wild type strain RN6390 revealed moderate infiltration of inflammatory cells, especially neutrophils in the alveolar lumen. There was not as much tissue necrosis and damage as was seen in pilot study and also the numbers of inflammatory cells were not as high as in the pilot study. More importantly, there were macrophages and plasma cells which indicated progression of inflammation toward the chronic stage (Fig.4.5.4.3). There were no visible changes in the hematoxylin and eosin stained gland tissue from the mutant strain challenged udder (Fig. 4.5.4.4). In the Gram-stained gland tissue from the RN6390 challenged udder, there were few Gram positive cocci as compared to the pilot study whereas no bacteria were observed in gland tissue from H330 challenged udder (Fig. 4.5.4.5).

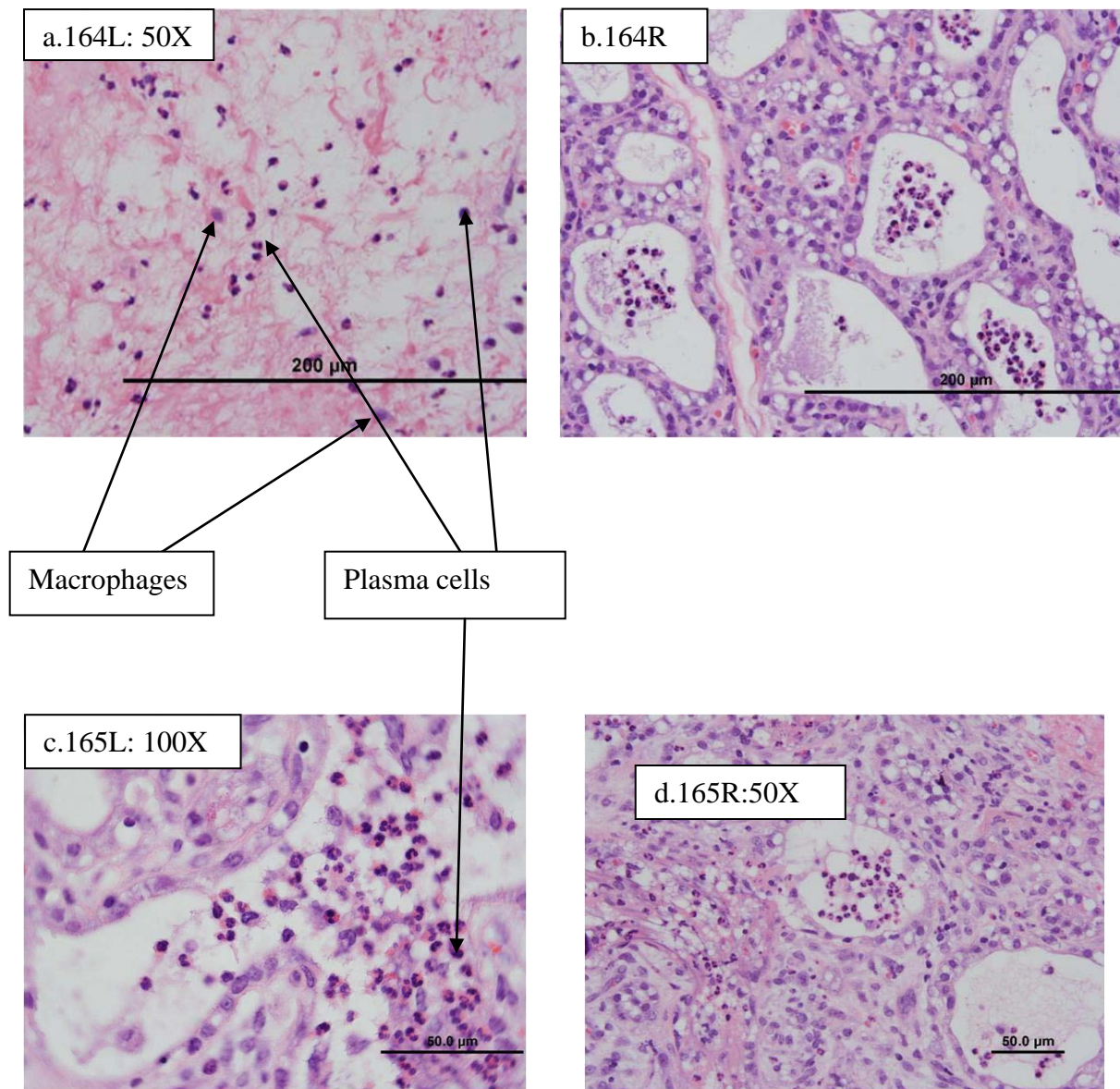


Figure 4.5.4.3a-d. Hematoxylin and eosin stained udder tissue from wild type strain RN6390 challenged Udders.

There were infiltrations of inflammatory cells mainly neutrophils which were visible in the alveolar lumen, however, their number was not as high as in the pilot challenge experiment. Also the tissue damage is moderate as compared to the pilot study. In panel a there are a few macrophages and plasma cells indicating progression toward chronic inflammation.

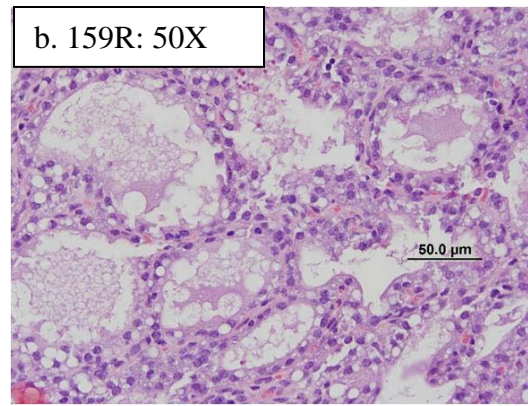
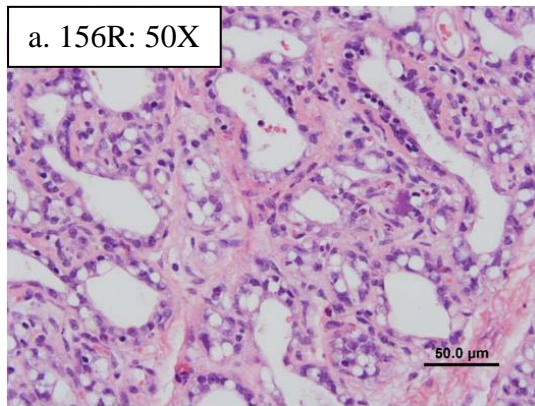


Figure 4.5.4.4a and b. Hematoxylin and eosin stained udder tissue from the mutant strain H330 challenged udder.

No detectable infiltration of inflammatory cells was observed

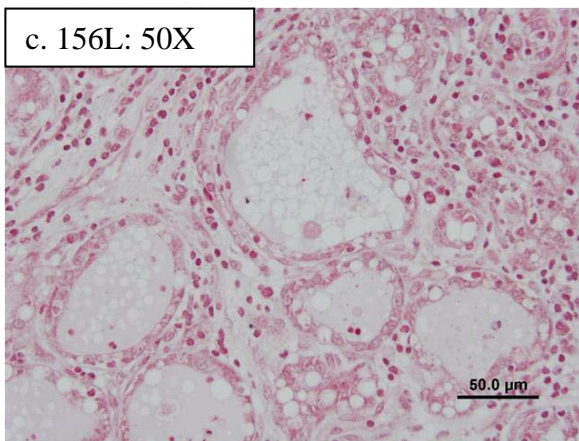
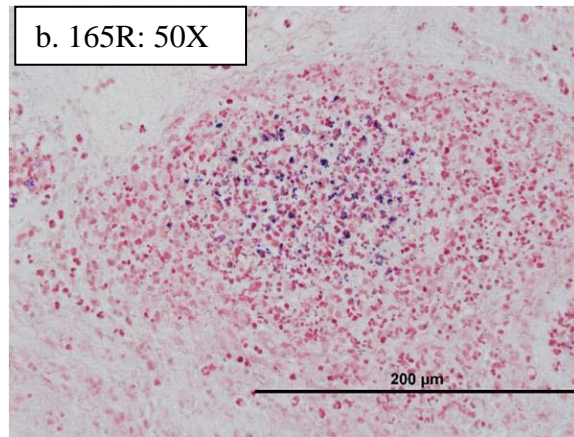
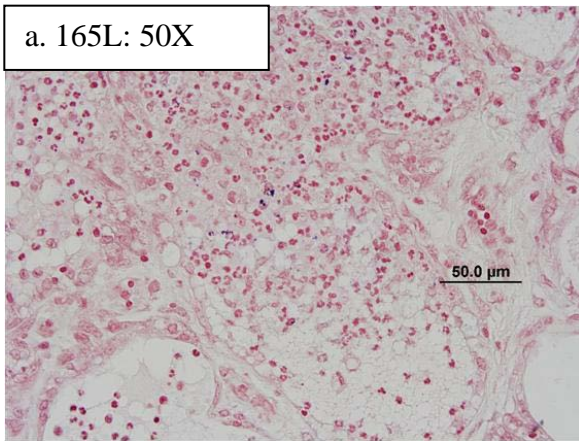


Figure 4.5.4.5a-c. Gram-stained gland tissue from wild type strain RN6390 and mutant strain H330 challenged udder.

Compared to the pilot study there were fewer Gram-positive cocci in the gland tissue (panel a and b) whereas there were no visible bacteria in gland tissue challenged by mutant strain (panel c).

#### 4.5.5. Discussion

*Staphylococcus aureus* has several virulence factors mainly categorized as cell surface structural components, secreted toxins, enzymes and proteases (Table 1.2.1.1). Some of these *S. aureus* cell surface structural components are considered adhesins which allow the bacteria to colonize the host through binding to specific and possibly non-specific ligands on the surface of host cells. These surface structural components are expressed during exponential growth of bacteria [50-54] and they are important for early attachment to the host cell surface which might allow the bacteria to escape the effects of natural and innate immune defenses through interference with recognition of epitopes or receptors. On the other hand, secreted virulence factors comprise toxins and enzymes (Table 1.2.1.1), which enable bacteria to invade and spread through the tissue of the host and they are collectively named invasins. In addition to these two broad categories of virulence factors *S. aureus* has many other mechanisms to adapt to the host environment such as immune evasion [56-58, 239, 529, 530], ability to attach and internalize into host cells and formation of small colony variants [531, 532]. The ability to attach *in vivo* [112-114] and *in vitro* [113, 115-122] and internalize [113, 115, 116, 124-130] into host cells allows the bacteria to escape from humoral immune responses and from antibiotics by being hidden in the intracellular area. This might allow *S. aureus* to cause chronic recurrent intramammary infections of cows and endovascular infections in human. Moreover, its ability to develop resistance to most recent antibiotics such as vancomycin and also the ability to form electron transport defective slow growing small colony variants that are less pathogenic but more difficult to treat makes it a more powerfully equipped pathogen that might be difficult to treat in the future. To combat such a versatile pathogen it is very important to understand the detailed mechanisms involved in the pathogenesis of disease. We hypothesized that staphylococcal GapC by the virtue of its surface localization, may function as an adhesin for *S. aureus* colonization of mammary gland tissue during early access of *S. aureus* into the intramammary area. We had tested the adhesion and invasion ability of *S. aureus* strain RN6390 and its isogenic GapC mutant strain H330 (RN6390 *gapC::tet*) into MAC-T cells *in vitro*. Our results showed that the number of adhered and/or invaded RN6390 strain into MAC-T cells was significantly higher than that of the isogenic GapC mutant (H330) strain. This suggests the involvement of GapC as a *S. aureus* cell- surface receptor for attachment to bovine mammary epithelial cells. The wild type strain

RN6390 killed MAC-T cells and was released into the supernatant after 2, 3, and 4 hs incubation in fresh media without antibiotics. The GapC mutant strain did not kill MAC-T cells since no viable counts were obtained after 2, 3 and 4 hs of incubation in fresh media without antibiotics. Although viable, there was no apparent intracellular growth of the GapC mutant and this might have contributed to the lack of cell lysis.

To determine whether the GapC mutant strain had a similar effect *in vivo*, we conducted a challenge trial on ovine mammary glands. Our results showed that the GapC mutant strain H330 was unable to cause mastitis in sheep whereas the isogenic wild type strain RN6390 caused acute febrile infection that lead to lameness, complete loss of appetite, bloody milk and bluish and gangrenous mammary gland within 24 hs. Although the viable count of RN6390 and its isogenic GapC mutant strains in the inocula were nearly the same, low numbers of GapC mutant strain were recovered from milk compared to the very high counts seen with its wild type strain. This might be because of rapid removal of the mutant strain by the host immune system. The somatic cell count of some animals included in this study were relatively higher prior to the challenge which was in the acceptable range for sheep in the early lactation period; however, the post challenge count from udders challenged with the wild type strain remained elevated while counts of the mutant strain dropped after 48 hs. Taking all our *in vitro* and *in vivo* results together we can conclude that GapC protein is an important virulence factor in the pathogenesis of *S. aureus* mastitis, at least in sheep.

#### **4.6. Regulation of Expression of *gapB* and *gapC* Genes.**

##### **4.6.1. Introduction**

There are two major families of regulatory elements controlling the expression of virulence determinants in *S. aureus*. They include the *sarA* (staphylococcal accessory regulator) protein family and the two component regulatory system (TCRS), which comprises *agr* (accessory gene regulator), *saeRS* (*S. aureus* exoprotein expression), *srrAB* (staphylococcal respiratory response), *arlSR* (autolysis related locus) and *lytRS* systems [185-187]. These

systems are sensitive to environmental signals and consist of a sensor histidine kinase and a response regulator protein. We hypothesized that the expression of *gapB* and *gapC* of *S. aureus* might be controlled by *agr* and *sar* loci which are known to be global regulators of virulence genes in *S. aureus*.

Microorganisms sense their immediate environment through their interactions with the environment and consequently respond to it by producing specific molecules that allow them to survive and multiply in that particular environment. Pathogenic microorganisms upon entry into host tissue induce transcription and translation of their virulence genes to produce virulence factors that allow them to survive and multiply in the harsh environment of host tissue. The expression of virulence genes in pathogenic microorganisms has been shown to be influenced by pH, oxygen tension, temperature, nutrient availability, growth phase, iron and other factors [533-536]. In *S. aureus* expression of virulence genes under *in vitro* condition by *agr* has been well characterized and regulation is known to be growth phase and cell density dependent. However, under *in vivo* condition the environmental stimuli that induce *sar*, *agr* and other regulatory genes transcription and translation are not well known. The environmental stimuli for *sar* expression is also not known. Few studies have shown that *agr* is repressed by alkaline pH and glucose [537, 538] and the production of  $\alpha$ -hemolysin is influenced by glucose, temperature and NaCl [539]. However, results from recent studies indicated that changes in *S. aureus* genes expression previously believed to be due to a glucose effect were instead due to a drop in pH as bacteria ferment glucose [536]. These authors also showed that many genes encoding extracellular virulence factors and genes involved in regulation of virulence factors are affected by a mildly acidic environment (pH 5.5). It has also been shown that the expression of  $\alpha$ -hemolysin, toxic shock syndrome toxin and staphylococcal protein-A was repressed by 1 M NaCl or 20 mM sucrose [540]. In another study the expression of staphylococcal respiratory response (*srrA*) and other unknown genes was shown to increase during anaerobic growth of *S. aureus* [541]. The role of external environmental factors as well as local microenvironmental factors especially under *in vivo* condition in the induction of virulence gene transcription and translation need further investigation.

To determine the role of *sar* and *agr* loci on the expression of *gapC* and *gapB* genes, total RNA was extracted from wild type strain RN6390 and its isogenic mutants *agrA*, *sarA* and



*sarA/agr* during exponential and stationary phases of growth. Using specific primers for *gapC* and *gapB*, cDNAs was synthesized by RT-PCR and used in qRT-PCR reactions to determine expression of *gapC* and *gapB* genes. The 16s rRNA gene is used as an internal control (section 3.2.6). We also decided to check whether protein expression correlated with mRNA expression and therefore, further analysed the expressions of GapB and GapC proteins under the same environmental conditions.

#### 4.6.2. Expression of *gapC* and *gapB* Genes in Wild type (RN6390) and its Isogenic *agrA*, *sarA* and Double (*sarA/agr*) Mutant Strains

The results for the exponential phase-cultures showed that the expression of *gapC* in the *AgrA* and *SarA/Agr* double mutant strains was 1000-fold down-regulated whereas the expression of *gapC* in the *sarA* mutant strain was 10,000 fold down-regulated. The expression of *gapB* was 1000 fold down regulated in the *AgrA* mutant strain whereas the expression of *gapB* in the *SarA* mutant and double mutant strains did not change. The results of the stationary phase cultures indicated that the expression of *GapC* and *GapB* in the *AgrA* mutant strain did not change whereas the expression of *gapC* and *gapB* in *SarA* and *SarA/Agr* mutant strains was 4-13- fold higher (Table 4.6.2.1).

Table 4.6.2.1. Expression of *gapC* and *gapB* genes in wild type RN6390 and its isogenic *agrA*, *sarA* and double (*sarA/agr*) mutant strains

Strain	Fold change				
		Exponential phase cultures		Stationary phase cultures	
	16s rRNA	<i>gapB</i>	<i>gapC</i>	<i>gapB</i>	<i>gapC</i>
RN6390	+ 1	+ 1	+ 1	+ 1	+ 1
<i>agrA</i> mutant	+ 1	- 100	- 100	+ 0.17	+ 0.77
<i>sarA</i> mutant	+ 1	+ 0.29	- 1000	+ 5.46	+ 4.44
<i>sarA/agr</i> mutant	+ 1	+ 0.29	- 100	- 5.50	+ 13.84

#### 4.6.3. Expression of *gapB* and *gapC* Genes under Different Environmental Conditions

4.6.3.1. Expression of *gapB* and *gapC* Genes under Different Environmental Conditions during the Exponential Phase of Growth.

### **i. Effect of pH**

In TSB the expression of *gapC* in SA16 and SA6390 was slightly increased at pH 5 and in SA6390 at pH 7.4 whereas *gapB* expression did not change in all strains. In whey, growth of all strains at pH 5 and pH 7.4 was poor in the first 7 hr of incubation. Due to this poor growth rate, it was not possible to isolate sufficient RNA. However, at 24 hrs all strains had grown to the stationary phase and thus I was able to purify RNA from these cultures (Table 4.6.3.1).

### **ii. Effect of Growth Media**

In TMG the expression of *gapB* and *gapC* increased in SA10 and SA12 and did not change in SA16 and SA6390, whereas in TMS their expression increased only in SA10 and remained unchanged in SA12, SA16 and SA6390. In whey, expression of both *gapB* and *gapC* increased in SA10 and decreased in SA16 and SA6390 (Table 4.6.3.1).

### **iii. Effect of Oxygen Tension**

In anaerobic growth conditions, the expression of *gapB* and *gapC* was slightly increased in SA12, SA16 and SA6390 and decreased in SA10 whereas in microaerophilic growth conditions, their expression increased in SA12 and SA6390, did not change in SA10 and decreased in SA16. In high CO<sub>2</sub>, expression of *gapB* and *gapC* increased in all strains except for a slight decrease of *gapC* expression in SA10 (Table 4.6.3.1).

Table 4.6.3.1. Expression of *gapC* and *gapB* genes under different environmental conditions after 7 hs of incubation (Late exponential growth phase)

The expression of *gapC* and *gapB* were measured at the late exponential phase of growth (7 hs) in different growth media (standard growth media (TSB), defined growth media (TMS and TMG) and whey), at different pH (5 and 7.4) and oxygen tension (aerobic, anaerobic, microaerophilic and high CO<sub>2</sub>) in different *S. aureus* strains SA10, SA12, SA16 and SA6390. The 16s rRNA gene is used as internal control gene. The fold changes in all conditions mentioned is calculated as compared to standard growth media (TSB).

Env. condition	Test media	Fold change in <i>gapB</i> and <i>gapC</i> gene expression in different <i>S. aureus</i> strains											
		SA10			SA12			SA16			SA6390		
		16s	<i>gapB</i>	<i>gapC</i>	16s	<i>gapB</i>	<i>gapC</i>	16s	<i>gapB</i>	<i>gapC</i>	16s	<i>gapB</i>	<i>gapC</i>
<b>pH</b>	TSB- pH5	1	.2	.6	1	+ 1.3	+ 1.1	1	.5	+ 3.2	1	0.1	+ 2.2
	TSB- pH7.4	1	.3	+ 1.1	1	.61	+ 1.4	1	0.3	.5	1	0.5	+ 2.7
	Whey- pH5	1	-	-	-	-	-	-	-	-	-	-	-
	Whey- pH7.4	1	-	-	-	-	-	-	-	-	-	-	-
<b>Growth media</b>	TSB (standard)	1	1	1	1	1	1	1	1	1	1	1	1
	TMG	1	+ 7.7	+ 8	1	+ 111	+ 123	1	.7	.8	1	1	+ 1.7
	TMS	1	+ 6.2	+ 4.9	1	.1	- 10	1	+ 1.2	.9	1	+ 1.2	+ 1.4
	Whey	1	+ 8.2	+ 8.2	1	+ 1.7	1.1	1	- 1000	.3	1	- 10	.1
<b>Oxygen tension</b>	Aerobic	1	1	1	1	1	1	1	1	1	1	1	1
	Anaerobic	1	0.1	.1	1	+ 1.4	+ 1.6	1	+ 1.4	+ 1.4	1	+ 2.8	+ 1.5
	Micro-aerophilic	1	0.8	+ 1.4	1	+ 2.8	+ 2.4	1	0.2	0.4	1	+ 2.6	+ 6
	High CO <sub>2</sub>	1	+ 362	.5	1	+ 4.2	+ 25	1	+ 1.1	1	1	+ 16	+ 11.3

#### 4.6.3.2. Expression of *gapB* and *gapC* Genes under Different Environmental Conditions during Stationary Phase of Growth.

##### i. Effect of pH

In TSB at pH 5 the *gapB* and *gapC* expression were increased in SA10 and SA16 respectively and decreased in other strains whereas in whey at pH 5 *gapB* was increased in SA10, SA12 and SA6390 and decreased in SA16. In whey at pH 5, *gapC* was slightly increased in SA16 and not changed in the other strains. In TSB at pH 7.4, *gapB* expression decreased in SA16 whereas expression of both *gapB* and *gapC* did not change in the other strains. In whey at pH 7.4, *gapB* expression increased in SA10, SA12 and SA6390 and decreased in SA16 however expression of *gapC* was not changed in any of the strains (Table 4.6.3.2).

## **ii. Effect of Growth Media.**

In TMG and TMS the expression of *gapB* was increased in SA10, SA12 and decreased in SA16 and SA6390. The *gapC* expression was increased in SA10, SA12 and SA16 and decreased in SA6390. In whey, *gapB* was up-regulated in SA10 and down-regulated in SA16 and SA6390 and remained unchanged in SA12. The *gapC* was down regulated in SA6390 and remained unchanged in SA10, SA12 and SA16 (Table 4.6.3.2).

## **iii. Effect of Oxygen Tension.**

In anaerobic growth conditions *gapB* expression was increased in SA10 and remained unchanged in SA12, SA16 and SA6390 whereas *gapC* was not changed except for a slight increase in SA16. In microaerophilic conditions *gapB* expression was increased in SA10, SA12 and SA16 and not changed in SA6390 whereas, *gapC* expression increased in SA12 and SA16 and remained unchanged in SA10 and SA6390. In high CO<sub>2</sub> conditions *gapB* was increased in SA10 and SA6390 and decreased in SA16 and not changed in SA12 whereas *gapC* was slightly up-regulated in SA10, SA16 and SA6390 and remained unchanged in SA12 (Table 4.6.3.2).

Table 4.6.3.2. Expression of *gapC* and *gapB* genes under different environmental conditions after 12 hs of incubation (Stationary growth phase).

The expression of *gapC* and *gapB* were measured at the stationary phase of growth (12 hs) in different growth media (standard growth media (TSB), defined growth media (TMS and TMG) and whey), at different pH (5 and 7.4) and oxygen tension (aerobic, anaerobic, microaerophilic and high CO<sub>2</sub>) in different *S. aureus* strains SA10, SA12, SA16 and SA6390. The 16s rRNA gene is used as internal control gene. The fold changes in all conditions mentioned was calculated as compared to standard growth media (TSB).

Env. condition	Test media	Fold change in <i>gapB</i> and <i>gapC</i> genes expression in different <i>S. aureus</i> strains											
		SA10			SA12			SA16			SA6390		
		16s	<i>gapB</i>	<i>gapC</i>	16s	<i>gapB</i>	<i>gapC</i>	16s	<i>gapB</i>	<i>gapC</i>	16s	<i>gapB</i>	<i>gapC</i>
pH	TSB- pH5	1	+ 13	0.9	1	.7	.3	1	- 10	+ 2	1	-1000	- 10
	TSB- pH7.4	1	+ 1.4	+ 1.1	1	.4	.7	1	- 100	.4	1	0.7	0.7
	Whey- pH5	1	+ 17	+ 1.4	1	1.5	0.8	1	- 10	+ 3	1	+ 73	0.9
	Whey- pH7.4	1	+ 57	1	1	+ 78	0.5	1	- 10	+ 1.6	1	+ 1.5	0.5
Growth media	TSB (standard)	1	1	1	1	1	1	1	1	1	1	1	1
	TMG	1	+ 46	+ 2	1	+ 3	+ 1.3	1	- 10	+ 831	1	.70	.8
	TMS	1	+ 22	+ 4	1	+ 3	+ 2	1	- 10	.6	1	- 10	- 10
	Whey	1	+ 2.2	.6	1	.8	.3	1	- 100	.6	1	- 10	-
Oxygen tension	Aerobic	1	1	1	1	1	1	1	1	1	1	1	1
	Anaerobic	1	+ 17	1.5	1	0.6	.4	1	- 10	+ 1.8	1	1	.4
	Micro-aerophilic	1	+ 46	0.6	1	+ 1.8	+ 29	1	+ 1.2	+ 3.2	1	0.8	0.5
	High CO <sub>2</sub>	1	+ 12	+ 1.9	1	.9	.8	1	- 10	+ 4.9	1	+ 2.6	+ 1.5

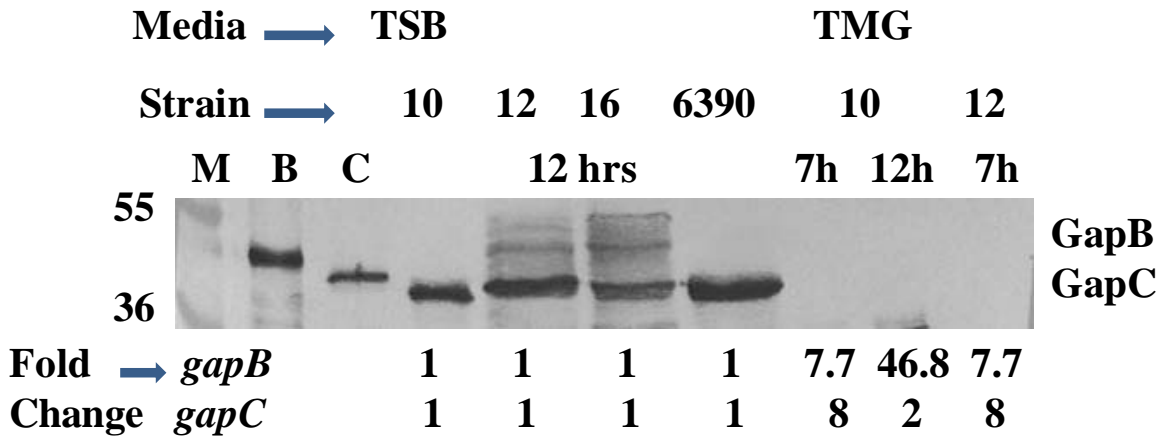
#### 4.6.4. Expression of GapB and GapC Proteins under Different Environmental Conditions

The expression of *gapC* and *gapB* genes of *S. aureus* strains SA10, SA12, SA16 and RN6390 were analyzed under different environmental conditions such as growth media, pH and oxygen tension at different time points. To determine whether the transcript expression level in each of these strains matched with the expression of the corresponding proteins the amount of cellular and extracellular GapB and GapC proteins were determined by western blots.

The results from this experiment showed that there were significant amounts of GapC proteins in the supernatants of growth media (Fig. 4.6.4.1a-f) as well as in the whole cell lysates (Fig. 4.6.4.2a-e). The GapB protein was not seen in the supernatants and whole cell lysates of the SA10 and RN6390 strains whereas its presence was seen in SA12 and SA16 under some

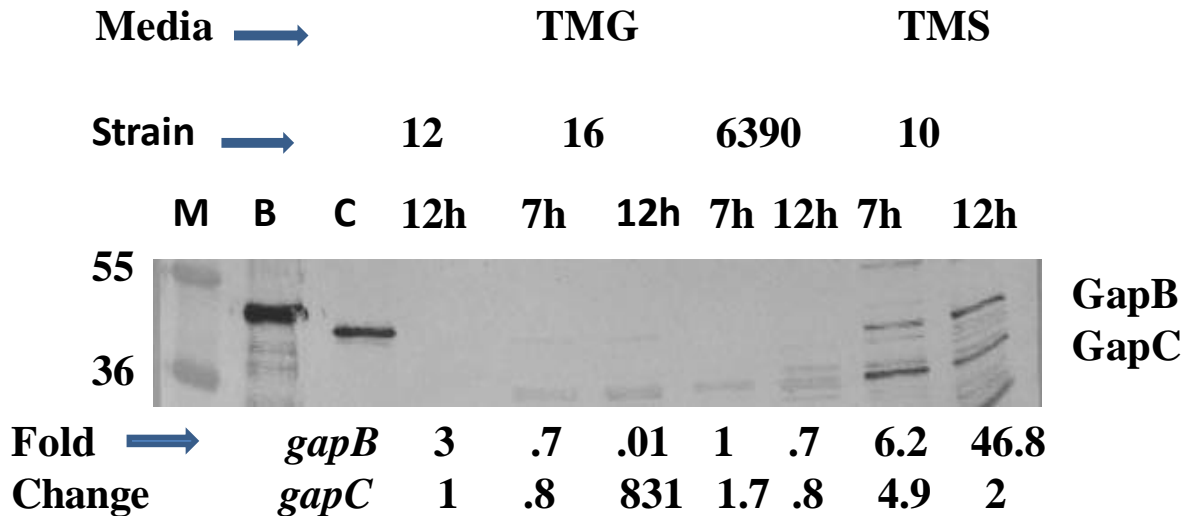
growth conditions. The amount of GapC protein in the supernatants and whole cell lysates of the tested strains did not correlate under some conditions with the amounts of messenger RNA previously determined under similar growth conditions. The amounts of GapC protein in the supernatant of growth media (Fig. 4.6.4.1a-f) were more consistent than the amount in the whole cell lysates (Fig. 4.6.4.2a-f). The amount of GapC protein in the supernatants of cells grown in TSB was consistently higher than its amount in other media tested (Fig. 4.6.4.1a). The amount of GapC protein in the whole cell lysates grown in TSB under microaerophilic condition was much more consistent than any other conditions tested (Fig. 4.6.4.2f).

Figure 4.6.4.1a-f. GapB and GapC proteins from the supernatants of cells grown in different media.



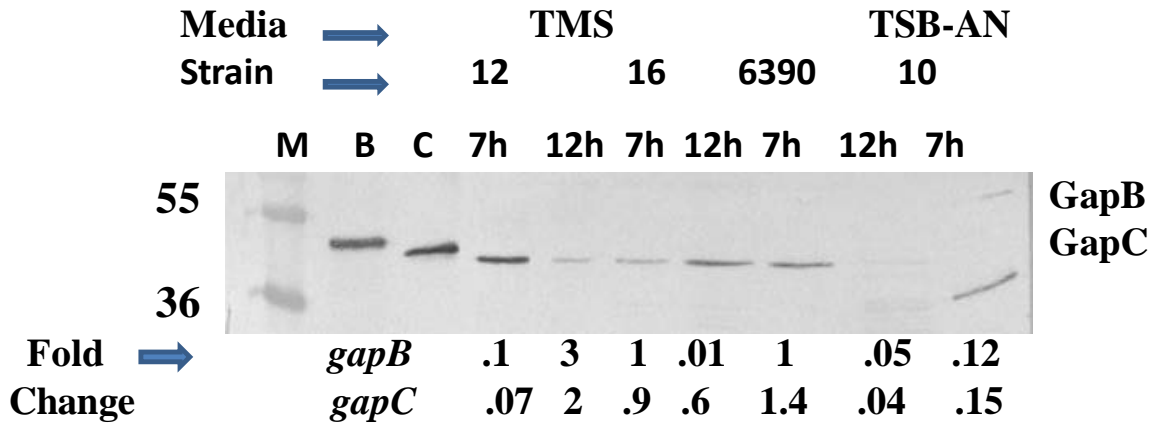
a. Supernatants from cells grown in TSB and TMG

M= Marker, B= GapB (positive control), C= GapC (positive control), TSB= Trypticase sy broth. TMG = Tris minimal glucose media.



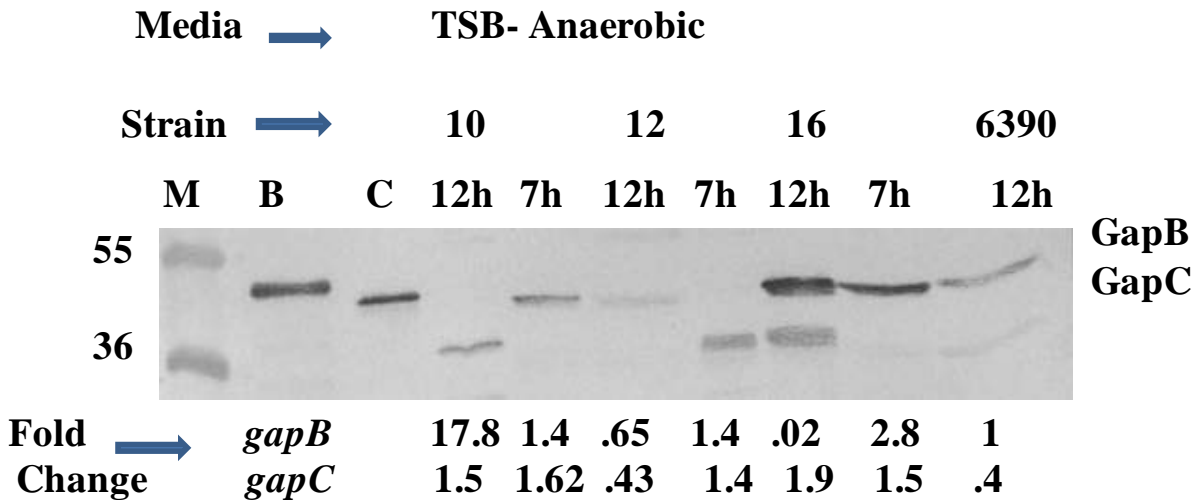
b. Supernatants from cells grown in TMG and TMS

M= Marker, B =GapB (positive control), C= GapC (positive control), TMS= Tris minimal succinate media, TMG = Tris minimal glucose media



c. Supernatants from cells grown in TMS and TSB-under anaerobic condition

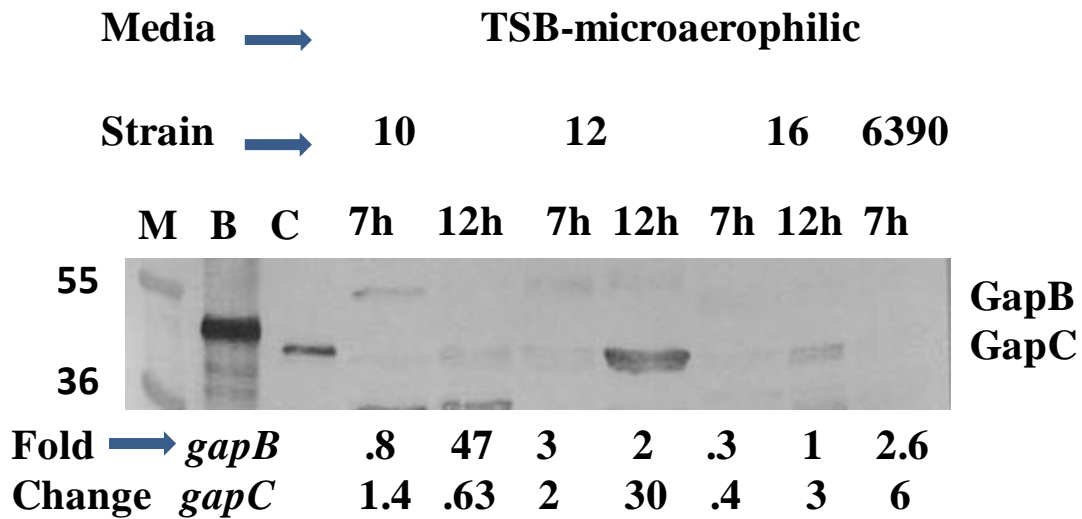
M= Marker, B =GapB (positive control), C= GapC (positive control), TMS= Tris minimal succinate media, TSB-AN = Trypticase soy broth, anaerobic conditions.



d. Supernatants from cells grown in TSB under anaerobic condition

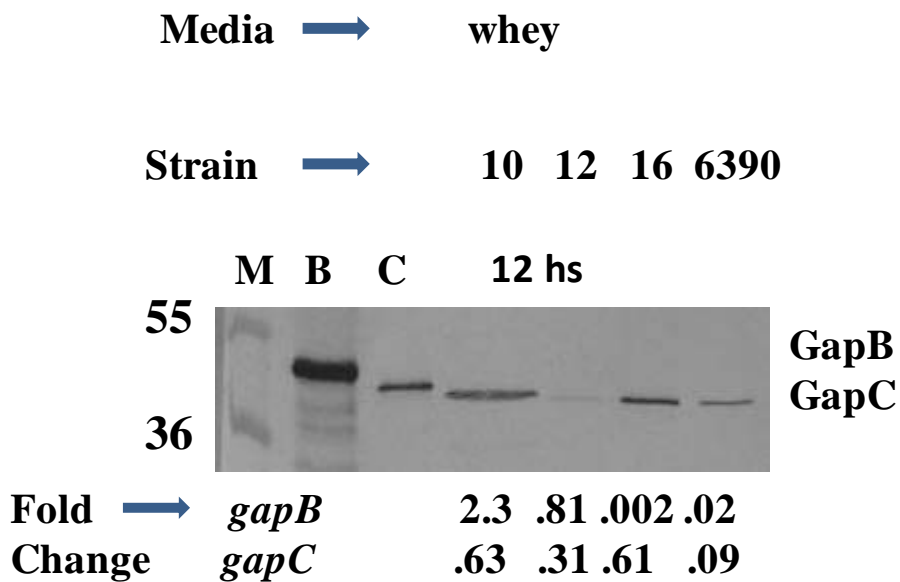
M= Marker, B =GapB (positive control), C= GapC (positive control), TMS= Tris minimal succinate media, TSB-Anaerobic = Trypticase soy broth, anaerobic conditions.





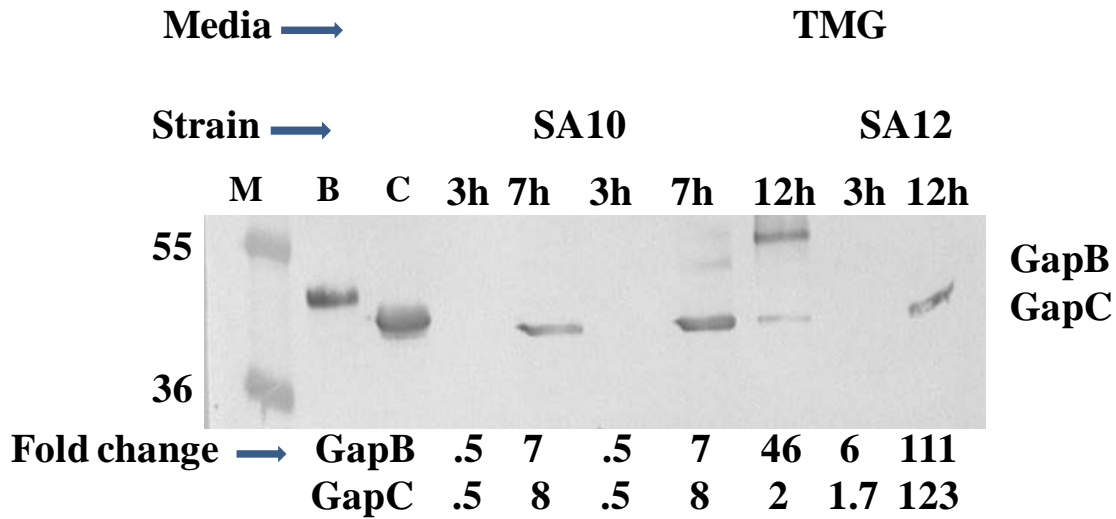
e. Supernatants from cells grown in TSB under microaerophilic condition

M= Marker, B =GapB (positive control), C= GapC (positive control), TMS= Tris minimal succinate media, TSB-microaerophilic = Trypticase soy broth, microaerophilic conditions.



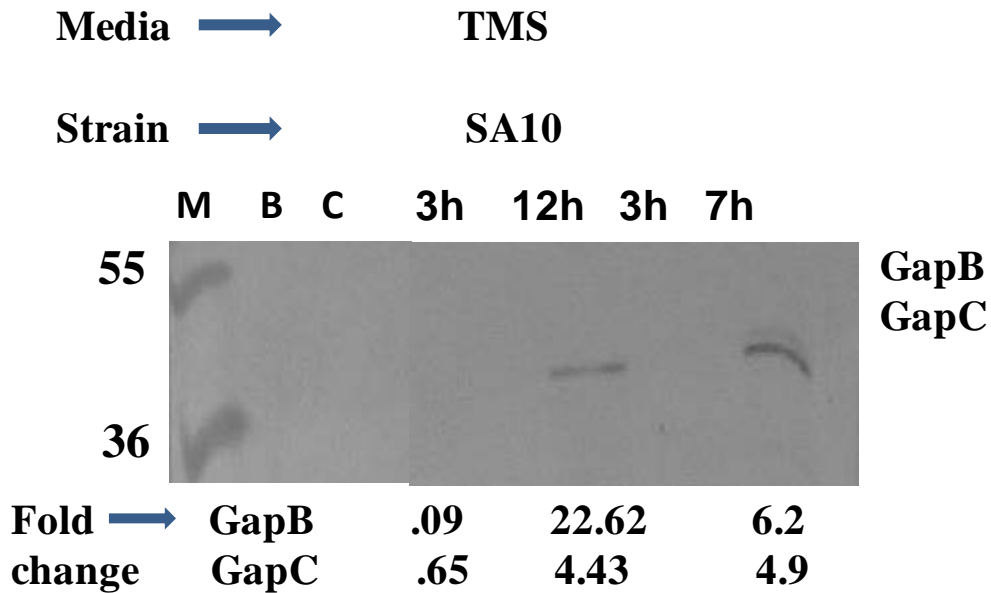
f. Supernatants from cells grown in whey

Figure 4.6.4.2a–e. GapB and GapC proteins in the whole cell lysate grown in different media.



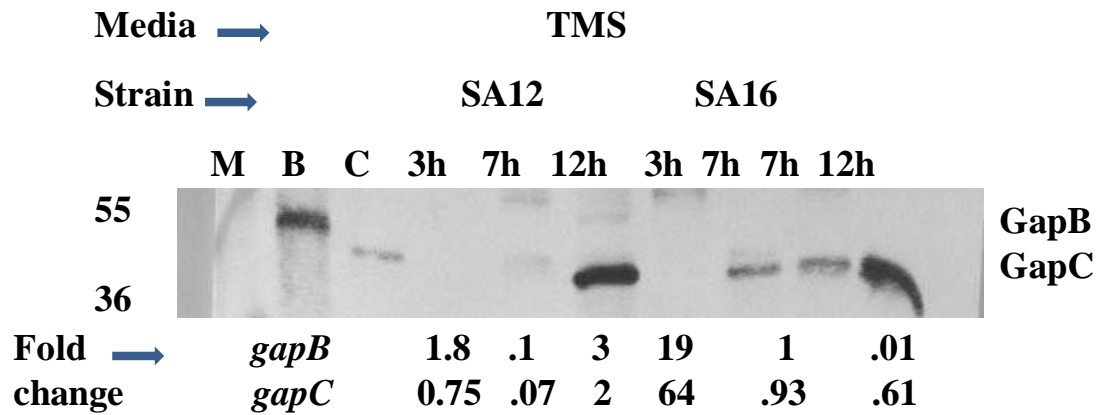
a. Whole cell lysate from strains grown in TMG

M= Marker, B= GapB (positive control), C= GapC (positive control), TMG= Tris minimal glucose media.



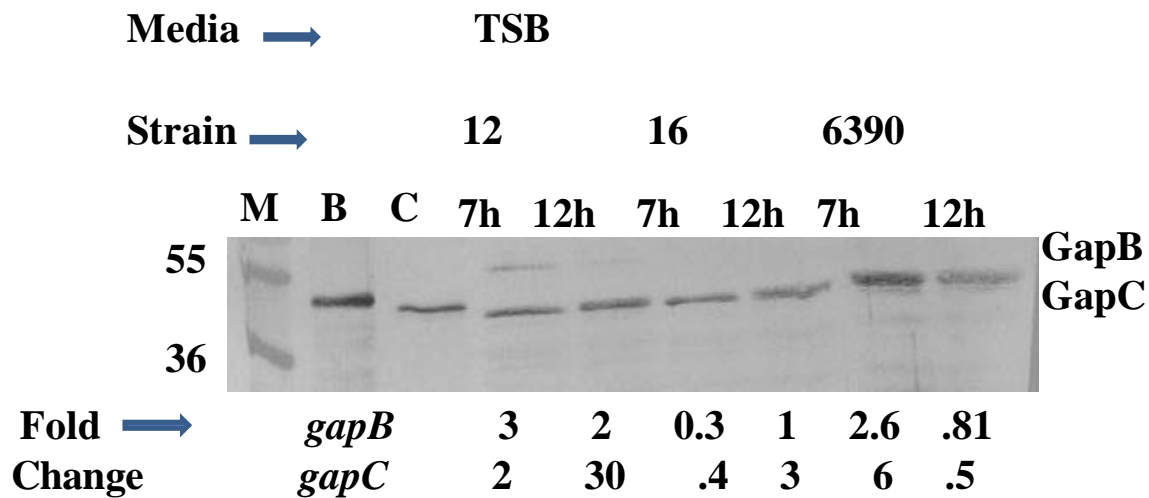
b. Whole cell lysate from strains grown in TMS

M=Marker, B=GapB (positive control), C= GapC (positive control), TMG= Tris minimal glucose media.



c. Whole cell lysate from cells grown in TMS

M=Marker, B=GapB (positive control), C= GapC (positive control), TMS= Tris minimal succinate media.



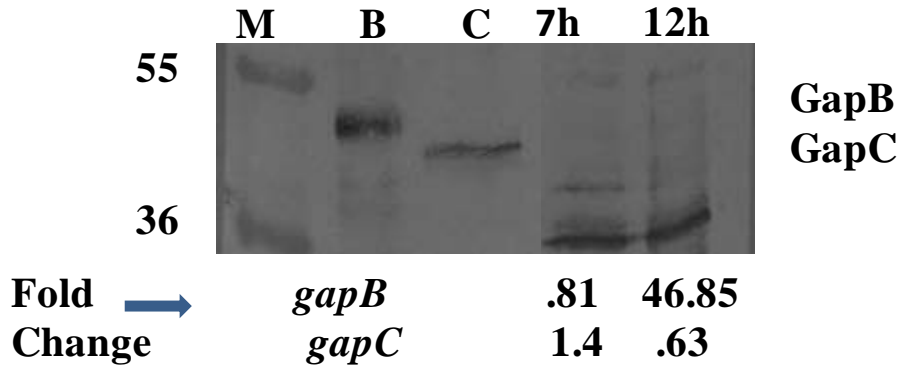
M=Marker, B=GapB (positive control), C= GapC (positive control), TSB: Trypticase soy broth.

d. Whole cell lysate grown in TSB- under microaerophilic condition

d.

**Media** → **TSB-under microaerophilic condition**

**Strain** → **SA10**



e. SA10 whole cell lysate in TSB under microaerophilic condition

M=Marker, B=GapB (positive control), C= GapC (positive control), TSB: Trypticase soy broth.

#### 4.6.5. Discussion

In general SarA promotes the expression of the genes for most of the *S. aureus* cell surface structural components and down regulates some of them, whereas *agr* down regulates the expression of genes for *S. aureus* cell surface structural components but up regulates the gene expression of secreted products [52, 181]. In the *agrA* mutant strain the expression of *gapB* and *gapC* were decreased by 1000 fold in exponential phase cultures whereas their expressions did not change in post-exponential phase cultures. AgrA is a response regulator in the *agr* two component regulatory system and its absence decreased expression of both GapB and GapC proteins on the surface of *S. aureus*, however, their expression during post exponential phase were not affected. This might be due to the presence of other regulatory mechanism such as SarA and its homologues as well as a sigma factor which are known to control expression of several genes during the post exponential phase of growth.

In a *sarA* mutant strain the expression of *gapB* was not affected whereas the expression of *gapC* was more than 10000 fold down regulated in exponential phase cultures, however, in stationary phase cultures both *gapB* and *gapC* were slightly up regulated. This might indicate that the expression of *gapC* is more influenced by *sarA* than that of *gapB*. Similar to the post exponential situation with the *agrA* mutant strain there might be other regulatory genes, for example, *agr* that compensate for the lost activity of *sarA* in the *sar* mutant strain.

In the double mutant strain (*sar/agr*) the expression of *gapC* is decreased by about 1000 fold in exponential phase cultures whereas *gapB* expression was not affected. In post exponential cultures the expression of both *gapB* and *gapC* were slightly up regulated. Again, this might indicate that the expression of *gapC* is more controlled by the *agr* and *sar* regulons than is *gapB*. Generally *gapC* gene expression is more affected by these universal regulators than is *gapB*.

The amount of GapC protein in the supernatant of tested growth media was relatively higher and more consistent than the amount seen in the whole cell lysate. The amount of GapC

protein in the supernatant as well as in the whole cell lysate of cultures in some of the growth media did not match with the corresponding amount of transcripts previously determined under similar environmental conditions. This can be due to several factors such as differences in the daily microenvironment, the presence of the proteins at different locations which might make its quantification inaccurate, as well as variation in the regulation of GapB and GapC under different environmental conditions. The amount of GapC protein in the supernatants of cultures in most of the media tested and especially in the whole cell lysate and supernatant of cells grown in TSB- under microaerophilic condition was consistent. The GapB protein was not seen in some strains such as SA10 and RN6390; however, it is visible in SA12 under some growth condition. In general GapC was detectable in all strains tested and it seemed to be conserved in most *S. aureus* strains compared to GapB. Overall there were no consistent correlations between strains as well as in individual strains under different environmental conditions tested. These findings were not surprising since *S. aureus* has multiple gene regulation mechanism which might vary among strains, environmental conditions and stages of bacterial growth [185, 187]

## 5. CONCLUSIONS

1. From two surface GAPDH antigens of *S. aureus* that appear to be conserved among pathogenic strains, a chimeric gene encoding the GapC and GapB proteins as a single entity (GapC/B chimera) was constructed as the basis for a multivalent vaccine.
2. Immunization with the recombinant GapC/B chimera resulted in significant humoral and cellular immune responses comparable to responses to the individual proteins.
3. Vaccination with *gapB*, *gapC* and *gapC/B* plasmid DNA did not result in a significant increase in cellular and humoral immune responses in C-57BL/6 mice
4. Vaccination with *gapB*, *gapC* and *gapC/B* plasmid DNA followed by booster vaccination with GapB, GapC and GapC/B proteins induced significant cellular and humoral immune responses in C-57 BL/6 mice.
5. Vaccination at the area drained by the supramammary lymph node resulted in better immune responses in the mammary gland of cows.
6. The GapC/B protein with VSA-3 as adjuvant was the best formulation in terms of inducing the highest immune responses in Holstein dairy cows.
7. The duration of GapC/B intramammary immune responses were approximately four months in Holstein dairy cows.
8. The number of wild type strain RN6390 that were attached and internalized into MAC- T cells was significantly higher than that of the isogenic GapC mutant strain H330.
9. The internalized wild type strain RN6390 started killing MAC-T cells 4 hs post infection whereas the isogenic GapC mutant strain H330 did not kill cells within this period of incubation.
10. The GapC mutant strain H330 was unable to cause mastitis in sheep whereas the isogenic wild type strain RN6390 caused acute febrile infection that lead to symptoms of mastitis within 24 hrs.
11. The GapC protein is an important virulence factor in *S. aureus* mastitis
12. The expression of the *gapB* and *gapC* genes in *S. aureus* is controlled by the universal virulence genes regulators, *agr* and *sar*.
13. The expression of *gapB* and *gapC* genes in different species of *S. aureus* was not consistent under different pH, growth media and oxygen tensions.

## 6. REFERENCES

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