School of Biomedical Sciences

Characterisation of Australian *Staphylococcus aureus* isolates of bovine mastitis origin, and development and evaluation of the potential of vaccines for prevention of mastitis using mouse as a model system

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Certification of Thesis

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Abstract

Mastitis is one of the most economically important diseases of dairy cattle in Australia causing an annual loss of more than $130 million per year. *Staphylococcus aureus* is the predominant contagious pathogen causing clinical bovine mastitis in Australia. The only effective treatment available against *S. aureus*-associated mastitis is treatment with antibiotics. However, due to emergence of antibiotic resistance by *S. aureus* strains and ability of the pathogen to develop biofilm in mammary gland, there is only 0-52% cure rate of mastitis in lactating animals. In the given scenario, there is an urgent need of an effective vaccine, which could facilitate prevention of bovine mastitis caused by *S. aureus*, particularly where herd management is failing.

The ultimate objective of this project was to develop an effective vaccine for prevention of bovine mastitis in cows taking into consideration the virulence factors and biofilm forming properties of *S. aureus* isolated from cases of bovine mastitis in cows. A total of 154 isolates of *S. aureus* from Victoria and Queensland were used in the study. Detection of biofilm formation by 154 *S. aureus* strains was accomplished using CRA and TCP methods which revealed the superiority of TCP method. TCP method could determine biofilm forming ability of 100% of the strains whereas only 31.17% of strains producing biofilm could be determined by CRA method. Amplification of *ica*A and *ica*D genes by conventional PCR showed that approximately 66.23% of the *S. aureus* isolates expressed *icaA* and *icaD* genes, respectively. However, only 2 (1.3%) strains were positive for the *bap* gene. No apparent relationship between biofilm formation by bovine *S. aureus* isolates and presence of *ica* loci, *bap* gene or *agr* types was evident in the current study, although a possible correlation between *blaZ* gene and penicillin resistance was evident. The antimicrobial susceptibility study of the 154 *S. aureus* strains against 12 different antimicrobial agents revealed that 100% of the strains were sensitive to cefoxitin and co-trimoxazole. Only 1 (0.65%) strain each was found resistant against cephalexin, tetracycline, vancomycin, rifampicin and linezolid, respectively. None of the *S. aureus* isolates were found to be resistant to methicillin as judged by the absence of *mecA* gene. Studies on the development of persistent antibiotic resistance by *S. aureus* revealed that the selected strains used in this study

developed resistance to more than one antibiotic after formation of biofilm. The resistance persisted as long as 4 weeks and then reverted back to antibiotic susceptibility when grown as planktonic cultures.

Detection of virulence factors of 154 *S. aureus* isolates of bovine mastitis origin has revealed that *clfA* (91.56%), *clfB* (92.86%), *spa* (87.7%), *fnbpA* (54.5%), *isdA* (98.1%), *isdB* (100%), *sdrD* (98.1%) and *sdrE* (95.5%) were the predominant MSCRAMMS, with α-toxin (94.16%), β-toxin (83.12%) being the predominant cytotoxins. A total of 4 (2.6%) isolates were found to carry the *pvl* gene. The potentially superantigenic enterotoxins produced by *S. aureus* isolates were *seh* (32.5%), *sec* (23.4%), *seg* (17.5%) and *sei* (13%).

Both genotyping and serotyping studies have revealed that 64.29% of *S. aureus* isolates were encapsulated with CP8 (31.82%) and CP5 (23.38%) being the predominant capsular types. Thirty percent of Australian *S. aureus* isolates from bovine origin were found to be non-typeable. There was a strong correlation between both the methods of CP typing for detection of CP types 1, 5 and 8, and the non-typeable *S. aureus* isolates.

A non-invasive mouse mastitis model, involving inoculation of *S. aureus* into mammary gland via mammary duct without traumatising the mammary gland surface epithelium or glands was established to enable evaluation of the immunogenicity and protective potential of vaccine candidates used in this study. Potential association of *ica*A and *ica*D genes and strength of biofilms of *S. aureus* in damage of mammary tissue was apparent in the investigation. Mice infected with encapsulated (CP8) strong biofilm forming *S. aureus* produced more severe mastitis lesions with evidence of necrosis in the mammary glands than in mice infected with CP8 encapsulated but weak biofilm forming *S. aureus* strain. This study also demonstrated the potential role of the pro-inflammatory cytokines, TNF-α in the tissue damage caused by *S. aureus* to the mammary glands. The level of TNF-α was significantly higher in sera sample of mice inoculated with the strong biofilm forming *S. aureus* than the weak biofilm forming strain even at 48 h post infection.

Mice infected with two different non-typeable *S. aureus* strains via intraductal route of mammary glands demonstrated minimal inflammatory infiltrates and tissue damage in the mammary glands. But the mice died within 24-36 h of post infection with *S. aureus*. Analysis of sera samples using collected from mice immediately before death showed significantly higher levels of pro-inflammatory cytokines particularly IL-6 suggestive of cytokine storm as the potential cause of death of mice. The potential role of IL-6 in the early mortality observed in mice was confirmed by the fact that administration of anti IL-6 and anti IL-6Rβ antibodies delayed the mortality for up to 13h 15 min. Severity of mastitis was also reduced in terms of clinical symptoms and bacterial loads from the mammary glands. The study highlights the potential importance of using anti-cytokine antibodies in the treatment of bovine mastitis and other *S. aureus*-associated infections.

Mice immunized with formalin killed whole cell of *S. aureus* residing in biofilm and delivered via i/mam route developed cell mediated immune response (CMI) as measured by IFN-γ as an indirect indicator of CMI. Mice immunised with this biofilm vaccine significantly reduced colonization of *S. aureus* in mammary gland, severity of clinical symptoms and tissue damage in mammary gland in comparison with the mice immunized with formalin killed whole cell of planktonic *S. aureus*. The planktonic vaccine administered by s/c route produced significantly higher humoral immune response than the biofilm vaccine. However, considering the tissue damage, clinical severity and colonisation of *S. aureus* in mammary gland the biofilm vaccine performed better when administered by i/mam route.

Protein A (*spa*), being prevalent in 87.7% of the isolates of *S. aureus* collected from bovine mastitis cases in Australia was used as a potential vaccine candidate against bovine mastitis using the mouse mastitis model established in this investigation. A moderate level of humoral antibody (IgG, IgG1 and IgG2a) response were produced in mice immunised by the s/c route, with lower level produced in mice immunised by the i/mam route. However, no significant difference in the CMI responses of mice immunised with planktonic vaccine was observed regardless of the route of immunisation. Furthermore, there was no difference in the clinical symptoms, bacterial loads or histopathological damage in mice immunised by s/c

versus i/mam route pointing out the unsuitability of Protein A as a component of any cocktail bovine mastitis vaccine.

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List of Abbreviations

AEC	Animal ethics committee
AMIR	Antibody mediated immune response
ANOVA	Analysis of variance
ATCC	American type culture collection
BBP	Bone sialo binding protein
BMCC	Bulk milk cell count
BP	Baird parker
CDMEM	Dulbecco's modified eagle's medium
CDS	Calibrated dichotomous sensitivity
CFU	Colony forming unit
CIP	Ciprofloxacin
CL	Cephalexin
Clf	Clumping factor
CMI	Cell mediated immune response
CMIR	Cell mediated immune response
CNA	Collagen adhesin
CoNS	Coagulase negative Staphylococci
CP	Capsular polysaccharide
CRA	Congo red agar
CSF	Colony stimulating factor
DNA	Deoxyriboneuclic acid
DNAse	Deoxyribonuclease
dPNAG	Deacetylated Poly-β-1, 6- linked N-acetylglucosamine
E	Erythromycin
ECM	Extracellular matrix

eDNA	Extracellular DNA
ELISA	Enzyme Linked Immunesorbent Assay
FAME	Fatty Acid Modifying Enzyme
FAO	Food and Agricultural Organization
FBS	Fetal bovine serum
Fn	Fibronectin
FnBP	Fibronectin binding protein
FOX	Cefoxitin
i/mam	Intra-mammary
i/p	Intraperitoneal
IB	Intermediate biofilm
IFN-γ	Interferon gamma
Ig	Immunoglobulins
IL	Interleukin
IMI	Intra-mammary infection
Kg	Kilogram
KPa	Kilopascals
L5	Left fifth pair of mammary glands
LZD	Linezolid
MH	Mueller Hinton
MHC	Major Histocompatibility Complex
min	Minute
ml	Milititre
mm	Milimeter
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules

MSSA	Methicillin sensitive *Staphylococcus aureus*
MT	Metric ton
MUP	Mupirocin
NA	Not applicable
NK	Natural killer
NS	Normal saline
NT	Non typeable
PBP	Penicillin binding protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDW	Primer did not work
pg	Picogram
PIA	Polysaccharide intercellular adhesion
PMN	Polymorphonuclear lymphocytes
PNA	Primer not available
PNAG	Poly-β-1, 6- linked N-acetylglucosamine
PSMS	Phenol soluble modulins
PVL	Panton valentine leucocidin
R	Resistant
R5	Right fifth pair of mammary glands
RD	Rifampicin
rEPA	Recombinant *Pseudomonas aeruginosa* exotoxin A
rSip	Surface immunogenic protein
rSUAM	Recombinant adhesion molecule of *Streptococcus uberis*
S	Sensitive
s/c	Subcuteneous
SAAC	Slime associated antigens

SAT	Slide agglutination test
SB	Strong biofilm
SCC	Somatic cell count
SE	Standard error
SE	Staphylococcal enterotoxin
SP	Surface polysaccharide
SXT	Co-trimoxazole
TCP	Tissue culture plate
TE	Tetracyclin
TEC	Teicoplanin
TM	Tube method
TSST	Toxic shock syndrome toxin
VA	Vancomycin
WB	Weak biofilm

Chapter 1: Literature review

1.1 Bovine mastitis

Mastitis (Mast: breast, itis: inflammation) is an inflammatory reaction of the mammary gland which may result from bacterial, thermal or physical causes. Mastitis leads to changes in the physical and chemical quality of milk and pathological changes in the mammary gland tissue. There are changes in the colour and consistency of milk from udder of cows suffering from mastitis. Milk from mastitis udder contains increased levels of serum albumin, immunoglobulins, somatic cells and decreased levels of casein, lactose and potassium (Auldist and Hubble, 1998). Pathological changes in the mammary gland of cow suffering from mastitis broadly include infiltration of inflammatory cells, abscess formation and degeneration of mammary tissue including necrosis (Gudding et al., 1984).

1.1.1 Classification of bovine mastitis

Mastitis can be classified as clinical or subclinical mastitis. There are visible abnormalities in milk and udder in clinical mastitis. The commonly observed abnormalities are swelling, heat, hardness and pain in udder and clots, blood, flakes in milk making it look watery. On the basis of level of severity, clinical mastitis can be further classified as sub-acute clinical, acute clinical, per-acute clinical and chronic clinical mastitis (Blood and Rodostits, 1989). In sub-acute clinical mastitis, there are very limited abnormalities including appearance of flakes in milk in initial ejections, slight swelling and reduction in milk production and microscopic changes in quality of milk. Acute clinical mastitis is always accompanied by redness, swelling, pain in the udder. There are prominent changes in the quality and quantity of milk with the manifestations of fever, anorexia and dehydration in cows. The most severe form of clinical mastitis is per-acute form where there is sudden death of the animal resulting from systemic infection and extensive damage of udder tissue. The chronic clinical form of mastitis results due to inadequate treatment of acute forms of mastitis.

In subclinical mastitis there are no any visible changes in udder or milk. The milk appears to be normal and the udder looks healthy. The only prominent observation is the sudden reduction in milk yield and quality of milk as judged by increased number of somatic cell count (SCC). Subclinical mastitis can be divided into two groups; infectious or non-infectious subclinical mastitis. The causative agent of infectious sub-clinical mastitis is a bacterial pathogen and there is elevation in the level of somatic cell count (SCC) in milk accompanied by reduction in milk production. The non-infectious sub clinical mastitis is due to other conditions such as age, trauma, season and there is absence of a pathogenic bacterium in milk. The SCC is elevated and there is reduction in milk yield from the udder. The udder is occasionally swollen and there is formation of fibrous tissue in the udder. There is absence of systemic changes in the cow and milk may contain some white flakes or bloody clots.

1.2 Economic implications of bovine mastitis

Mastitis is one of the most economically devastating diseases of dairy cattle particularly for the back yard farmers in developing world, with different levels of economic losses reported by different countries. As it is a production related disease the loss associated with bovine mastitis are reduction in milk production, poor quality of milk and milk-products, preventive measures of mastitis, treatment cost including cost of drug, veterinary services, culling of infected cows, cost of labour (Hogeveen and Osteras, 2005). Per-acute cases of mastitis may lead to sudden death of cows causing maximum loss to dairy farmers (Menzies et al., 1995). The total annual loss due to bovine mastitis is estimated to be $35 billion all over the world (Ratafia, 1987). The cost of mastitis involving various factors may differ from country to country, but the economic principles involved for loss are similar in all countries (Hogaveen and Osteras, 2005; Halasa et al., 2007). A comparative data compiling the loss due to mastitis in different countries is presented in Table 1.1.

1.3 Epidemiology of bovine mastitis

1.3.1 Etiology of bovine mastitis

Major advances in the fields of animal breeding, animal nutrition and husbandry practices have played a significant role in increasing the global milk yield over the

Table 1.1: Comparative Data on loss due to mastitis in different countries

Rank	Country	Milk production (million MT)*	Losses due to mastitis			
			Annual (US$ equivalent, million)	Per cow per year (US$ equivalent)	Year of estimate	Reference
1	India	107.03	1150-1200		2001	Dua, 2001
			320		1994	Singh and Singh, 1994
2	United States of America	85.88	1700-1800	185	1996	Bramley et al., 1996
3	China	38.51		160	1991	Fang et al., 1991
4	Pakistan	33.61	NA	NA		
5	Russian Federation	32.33	NA	NA		
6	Brazil	30.00		95-142	2005	Swinkels et al., 2005
7	Germany	29.20		285	2001	Hamann, 2001
8	France	22.65		102.42	2006	Fourichon et al., 2001
9	New Zealand	15.67		67.70	1993	Holdaway, 1993
				72.56	1990	Scharenraad and Dijkhuizen, 1990
10	United Kingdom	13.24	NA	191.26	1999	Bennett et al., 1999
				75-131	1993	Esslemont and Peeler, 1993
11	Poland	12.45	NA	22-257	2011	
12	Turkey	11.61	NA	72.56	1990	
13	Netherlands	11.47				Hogeveen et al., 2011
						Scharenraad and Dijkhuizen, 1990
14	Ukraine	11.36	NA			
15	Mexico	10.55	NA			
16	Italy	10.70	NA			
17	Argentina	10.37	NA			
18	Australia	9.39	130	200	2011	Dairy Australia, 2011
19	Canada	8.21		19.6	1996	Dekkers et al., 1996
20	Japan	7.91	NA			

NA=Not available *Combined output from cows and buffaloes as of 2009 (FAO:http://faostat.fao.org/site/339/default.aspx).

last 2 decades (Shook, 2006) meeting the overwhelming demand for milk and milk products. There have been continuous changes in the predominance of etiological of mastitis (Zadoks and Fitzpatrick, 2009), greater understanding of the host responses to intra-mammary infections (Oviedo-Boyso et al., 2007; Attalla et al., 2010) and treatment regimens leading to adoption of various control and prevention measures.

Regardless, the problem of mastitis continues to pose the greatest challenge to the dairy industry worldwide. As many as 137 species and subspecies of microbes can be associated with mammary infection (Watts, 1988). The predominant causal organism is bacteria; however, instances of Mycoplasma, yeast and algal infection have also been reported (Wilson et al., 1997; Barkema et al., 1998; Fox et al., 2005). Mycoplasma species associated with mastitis in cows are *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Mycoplasma canadense*, *Mycoplasma californicum and Mycoplasma alkalescens*. Prototheca, a group of yeast-like micro-algae that have been described as a cause of mastitis in Japan, Europe and North and South America (Ruth et al., 2011).

Mastitis in dairy herds is generally of two types: Environmental mastitis and contagious mastitis. They differ in their causal factors.

Environmental mastitis is caused by the coliform bacteria that enter through the teat canal contacted through contaminated environment (Boyer, 1997). As coliform bacteria are normal inhabitants of soil, digestive tract and manure, they multiply in contaminated bedding and a substantial proliferation in their numbers (to the tune of 1000,000 or more per gram of bedding) increase the probability of infection and clinical mastitis (Bradley and Green, 1997). Coliforms particularly *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumonia* and *Serratia marcescens* and a *Streptococcus sp*, *Streptococcus uberis*, are the chief organisms found to cause environmental mastitis. The cases of environmental mastitis however rarely exceed 10% of the total cases even though there is an increasing body of evidence suggesting the contrary (Boyer, 1997; Bradley and Green, 1997; Edmondson, 1997; Bradley and Green, 2001; Bradley et al., 2007; Dairy Australia, 2011) particularly with regards to mastitis associated with *S. uberis* infection.

Contagious mastitis is caused by bacteria that thrive on the skin of the teat or inside of the udder and is transmitted by milking. The pathogens mainly associated with contagious mastitis are *Staphylococcus aureus*, *Streptococcus dysgalactiae and Streptococcus agalactiae*. However, the most recognised pathogen in majority of clinical and subclinical mastitis cases (40.5%) in most countries is *S. aureus* (Workineh et al., 2002; Middleton, 2008; Pereira et al., 2011). These bacteria are of

immense importance because though their prevalence seldom exceeds 25% in intra-mammary infections; their effect clearly affects the milk quality premiums and account for a considerable portion of clinical cases (Owens et al., 1988). They are considered the emerging pathogens of bovine mastitis as they are the most commonly isolated ones (Pyolara and Taponen, 2009; Pereira et al., 2011). In cattle, mastitis is the only disease associated with *S. agalactiae* infection and transmission within the herds occurs due to inadequate hygiene in the farm (Ruth et al., 2011). *S. aureus* is a versatile organism and it also demonstrates strain-specific associations with somatic cell count, milk yield, biofilm production, clinical signs, persistence and treatment response (Ruth et al., 2011).

In addition to *Staphylococcus sp*, *Corynebacterium sp* constitute some of the emerging pathogens causing bovine mastitis. *Corynebacterium bovis* is frequently isolated from milk in many dairy farms and causes moderate inflammation of the mammary gland (Hommez et al., 1999; Haltia et al., 2006). These infections result in a slight increase in bulk tank somatic cell counts, changes in the composition of milk, sudden reduction in milk production and clinical mastitis (Harmon, 1994). Four species of non-lipophilic *Corynebacteria* found to cause clinical and sub-clinical mastitis are *C. amycolatum, C. ulcerans, C. pseudotuberculosis, and C. minutissimum* (Hommez et al., 1999). Other species of *Corynbacterium* isolated from cases of clinical mastitis in sheep are *C mastiditis and C. camporealensis* (Fernandez-Garayzabal, 1997a, b).

Recent studies have revealed that coagulase negative staphylococci (CoNS) isolated from teat skin, teat canal, and vagina as well as from the coat and the nostril comprises an area of interest as causal organisms of bovine mastitis (Pittkala et al., 2004; Tenhagen et al., 2006). Mastitis in heifers at calving is mainly caused by CoNS. More than 50 species and subspecies of coagulase negative staphylococci are included in this group (Pyolara and Taponen, 2009), *Staphylococcus epidermidis, Staphylococcus simulans, Staphylococcus saprophyticus, Staphylococcus hyicus, Staphylococcus warneri, Staphylococcus chromogenes, Staphylococcus scrimi* and *Staphylococcus xylosus* being the commonly encountered species of CoNS in bovine mastitis (Rupp et al., 2000). The various species of CoNS isolated from bovine

mastitis cases show varied pathogenicity, antimicrobial susceptibility and virulence factors (Zadoks and Schukken, 2006; Taponen and Pyolara, 2009).

Mastitis caused by fungi and yeast is uncommon. The fungi commonly associated with mastitis are *Candida sp, Trichosporon sp, Saccharomyces sp* and *Aspergillus sp* (Chahota et al., 2001). A very rare case of mastitis caused by yeast like fungus, *Geotrichum candidum* has also been reported (Chahota et al., 2001). Though the incidence of mycotic mastitis is very low, serious problems may arise when it occurs in enzootic form (Sharma et al., 2012).

1.3.2 Factors affecting occurrence of bovine mastitis

1.3.2.1 Host factors associated with bovine mastitis

The large number of predisposing factors that contribute to the emergence of mastitis in dairy cattle may be physiological, genetic, pathological or environmental (Sordillo, 2005) are described below:

1.3.2.1.1 Age of the cow

It has been demonstrated that occurrence of mastitis in infected quarters increases with age in cows (Emanuelson and Persson, 1984; Harmon, 1994; Busato et al., 2000; Sharma et al., 2007; Sharma and Maiti, 2010), being the highest at 7 years of age (Schukken et al., 1989). This may be due to an increased cellular response to intramammary infection or due to permanent udder tissue damage resulting from the primary infection. Efficient innate host defence mechanisms of the younger animals are one possibility that makes them less susceptible to infection (Dulin et al., 1988). However, at least one study conducted using 4133 cattle including both cross-bred and non-descriptive breeds, revealed the highest risk of occurrence of mastitis to be between the ages of 4-6 years, followed by the age group between 2-4 years, with the least occurrence noted between 6-8 years of age (Mahajan et al., 2011). Interestingly it was noted in this study that the cross bred animals were 2.55 times more susceptible to mastitis than the non-descript or indigenous ones.

1.3.2.1.2 Inherited features of the bovine

Various genetic traits may also have a considerable impact upon the susceptibility of the animal to mastitis. These genetic traits include the natural resistance, teat shape and conformation, positioning of udders, relative distance between teats, milk yield and fat content of milk. High milk yielders with higher than average fat content are reported to be more susceptible to mastitis (Grohn et al., 1990; Rajala and Grohn, 1999; Lactation Resource Library, 2009). The conformation of the udder and shape of the teat are inherited characteristics that may also affect susceptibility to mastitis. Cows with elongated teats are more vulnerable to mastitis infection than cows with inverted teat ends (Rathore, 1976; Seykora and McDaniel, 1985). Broad udders, lower hind-quarters and teats placed widely help the infectious agent and should be selected against (Thomas et al., 1984). Another important predisposing factor for mastitis is super numerous teats, which provide additional reservoirs for potential pathogens leading to manifestation of mastitis.

1.3.2.1.3 Stage of lactation:

The incidence of mastitis is reported to be higher immediately after parturition, early lactation and during the dry period, especially the first 2-3 weeks (Schukken et al., 1989; Ellis, 2005; Corbett, 2009; Fadlelmula et al., 2009) due probably to increased oxidative stress and reduced antioxidant defence mechanisms during early lactation (Sharma et al., 2011). An increase in somatic cell numbers or count (SCC) which is mainly neutrophils is observed immediately after parturition, which remains high for a few weeks irrespective of the presence or absence of infection (Plastridge, 1958; Bradley and Green, 2005). This increased SCC is the cow's natural first line of defence to prepare for the onset of the new lactation. Relatively recent studies have revealed that cows in late lactation always show a higher than average SCC than that seen at other stages of the lactation period (Peeler et al., 2000), potentially representing increased subclinical infection, leading to a fall in milk production. Milk somatic cells are primarily leukocytes or white blood cells including macrophages, lymphocytes and neutrophils, which serve as a defence mechanism to fight infection and assist in repairing damaged tissue.

1.3.2.1.4 Mammary regression:

There are significant functional changes in the udder during the early and late lactation and dry period, which affect the cow's susceptibility to infections (Giesecke et al., 1994; Capuco et al., 2003). Lactating cows under stress show premature mammary regression. Such a condition compromises udder's natural defence mechanisms (Giesecke et al., 1994; Capuco et al., 2003) leading to invasion of the teat canals by potential pathogens. The same condition prevails during the healing process of lesions because the resistance to causal agents remains less effective.

1.3.2.2 Environmental factors associated with mastitis

1.3.2.2.1 Milking machine

Extraneous factors such as the milking habits of farmers and faulty milking machines favour the pathogens to gain access to mammary gland and proliferate, potentially leading to mastitis (Mein et al., 2004). In farms where machines are employed for milking it is important to maintain physiologically optimal pressure [50 kPa for most machines], because pressures in excess of this may lead to injury in the teat (Blood and Rodostits, 1989). Fluctuations in the pressure due to inadequate vacuum reserve must be avoided to prevent occurrence of mastitis. Proper installation as well as the correct maintenance of milking machines is important to avoid an inadequate vacuum level, teat and tissue damage and incomplete milking (International Dairy Federation, 1987). The vacuum level created by the vacuum pump is another important factor for complete and high quality milking. Experiments have shown that a teat subjected to a vacuum level of 10.5-12.5 inches at the time of peak milk flow results in rapid, complete and high quality milk yield, and the teat suffers minimum physical pressure (Jones, 2009a). Two-chambered teat cups are found to be better than single chambered teat cups in regard to achieving complete milking as well as fewer incidences of teat injuries (Mein and Schuring, 2003). However, there is a report of increased risk of both contagious and environmental mastitis causing pathogen due to machine induced changes, which widen the orifice of the teat canal in cows (Mein et al., 2001).

1.3.2.2.2 Nutrition

The quality and plan of nutrition appears to be an important factor that influences clinical manifestation of mastitis in heifers and cows (Heinrichs, 2009) although no

relationship between the incidence of mastitis and either high energy or high protein feed in cows has been reported (Madsen and Nielsen, 1981; Rodenburg, 2012).

Vitamin E is one of the important supplements in dairy feed to boost the immune response of cows (Spears and Weiss, 2008) as it has been reported to enhance the neutrophil function as well as the phagocytic properties of neutrophils after parturition (Hogan et al., 1992). Vitamin E is often combined with selenium, which acts as an anti-oxidant by preventing oxidative stress (Chamberlain and Wilkenson, 1996; Mustacich and Powis, 2000). A number of investigations have demonstrated that neutrophils of selenium fed cows are more effective at killing mastitis causing microorganism such as *E. coli* than those not supplemented with selenium (Underwood and Suttle, 1999).

Beta-carotene and Vitamin A have also been found to be effective in preventing the occurrence of mastitis, most probably due to their antioxidant and immune-enhancing properties and contribution mucosal surface integrity of the mammary gland respectively (Sordillo et al., 1997). Zinc and copper are also important nutritional elements that contribute mammary gland health by promoting cellular repair, wound healing and reduction in SCC (Prasad et al., 2004; Bruno, 2010) aided by increases in metallothionein synthesis with antioxidant potential. Various studies have shown that feed supplemented with copper and fed to heifers reduces the severity of subclinical mastitis as well as clinical mastitis induced by *Escherichia coli* (Scaletti et al., 2003; Upadhayay et al., 2009).

1.3.2.2.3 Weather and Climate

The incidence of mastitis is greatly influenced by the weather conditions and prevailing climatic conditions. Heat, humidity, cold and draught are the important predisposing factors (Jones, 2001; Dhakal et al., 2007; Akyuz et al., 2010; Reneau, 2012). A higher incidence of mastitis has been reported to occur particularly during summer rainy months (Ribeiro et al., 2001; Akyuz et al., 2010; Reneau, 2012; Sentitula et al., 2012); as heat and humidity increases, so does the bacterial multiplication as well as the load of pathogens in the environment (Godden et al., 2003). Conversely, an alternative study has reported a higher incidence of coliform

mastitis during the cold months of the year when the temperature was reported to be less than 21°C (Shathele, 2009; Ranjan et al., 2011).

1.3.2.2.4 Hygiene

The important sources of bacteria causing mastitis are the infected udder, teat canal, bedding material, manure, water, feed and milking machine. Environmental mastitis caused by the coliform bacteria is generally transmitted through contaminated environment (Boyer, 1997). Various researchers have demonstrated that there is incidence of clinical mastitis in cows reared in dirty barn and bedding material containing increased colony counts (Bramley and Neave, 1975). Coliforms and environmental streptococci enter into the mammary gland during the dry period and produce clinical mastitis during lactation. Contagious mastitis causing organisms are spread from infected quarter to healthy ones during milking via milker's hands, clothes used to wipe udders, teat cup liners and milking machine. Splashes and aerosol of milk also play indirect role to transmit infection. A study conducted in NIRD, England demonstrated that 50% of milker's hands before milking and 100% of milker's hands after milking were contaminated with mastitis causing organisms (Dodd et al., 1966).

1.4 Mammary gland immunity and bovine mastitis

In the last two decades there has been immense development in the area of mammary gland immunity and its function. Identification of leukocyte subpopulation by flow cytometry analysis, studies of bovine cytokines and their roles mammary gland defence, development of recombinant cytokines as disease control tool has given new dimension to the area of mammary gland immunity. Mammary gland immunity can be defined as the protection developed by the body against various infectious agents by using variety of immune and non- immunological factors (Sordillo and Streicher, 2002). The immune components are capable of recognizing and differentiating the foreign agents and body's own molecules and upon recognition, the infection is attempted to eliminate by both cellular and soluble factors. The non-immune components comprise various protection mechanisms which are non-specific to particular causative agent.

1.4.1 Immune defence mechanisms of mammary gland

There are two distinct forms of defence mechanisms which protect the mammary gland against variety of microorganisms. They are innate or nonspecific immunity and acquired or specific immunity. Innate immunity is activated at the early stage of infection by various stimuli and most of the infections are eliminated if this immunity is functioning adequately. However, it does not recognize any specific antigenic determinant of the causative pathogen. The various components of innate immunity are teat canal, neutrophils, macrophages, natural killer cells and cytokines. The acquired immunity is triggered when the innate immunity fails to eliminate the infection. The major functions of acquired immune system are recognition of specific pathogen by antigenic presentation process, elimination of the pathogen or pathogen infected cells and development of immunological memory by T cell receptors for future infections by the same pathogen. Due to development of immunological memory, there will be faster and stronger response against the pathogen in its subsequent encounters.

The mammary defence mechanisms can be further characterized as anatomical, cellular and soluble factors against variety of mammary gland infections which are discussed below.

1.4.1.1 Anatomical defence mechanisms

Teat canal plays an important role in the defence mechanism of udder against variety of pathogens. Teat canal, a cylinder shaped body opening prevents leakage of milk and gain entry of microorganisms into the udder. The sphincter muscle of teat keeps the teat orifice tightly closed to prevent penetration of any mastitis causing bacteria and thus acts as a mechanical barrier. The teat canal is lined by a stratified squamous epithelium or keratin which prevents or limits the entry of bacteria into the teat cistern by its variety of physical, chemical, bacteriostatic and bactericidal properties. Accumulation of keratin acts as a physical barrier to the pathogens and in non-lactating cows the teat canal is completely blocked to prevent entry of any foreign substances (Nickerson, 1987). The esterified and nonesterified fatty acids component of keratin layer including myristic acid, palmitoleic acid, linoleic acid exhibit bacteriostatic effects. Certain cationic proteins present in keratin exert bacteriostatic

effect which alter cell wall of invading bacteria after binding electrostatically and lead to death of the organism (Hogan et al., 1987).

1.4.1.2 Cellular defence mechanisms

If the mastitis-causing bacteria can invade the teat canal, they need to evade the cellular defence mechanism of mammary gland. This mechanism consists of neutrophils, macrophages and lymphocytes which lead to either innate or acquired immunity (Sordillo and Streicher, 2002).

1.4.1.2.1 Neutrophils

Neutrophils are the predominant cell type present in mammary tissue during early stage of infection. There is constant supply of neutrophils in sterile mammary gland as milking stimulus induces migration of neutrophils from blood to the mammary tissue (Paape et al., 1992). But after reaching the alveolar lumen and ingesting on fat and casein, they lose the phagocytic property and die and these dead neutrophils are removed by milking and replaced by healthy neutrophils (Paape and Wergin, 1977). In healthy animal udder the production and destruction of neutrophils are regulated in such fashion that there is constant maintenance of particular number of neutrophils in blood, milk and mammary tissue (Jain, 1986). There is rapid migration of neutrophils once there is infection in the mammary gland which is mediated by the chemotactic components released by the causative agent and other variety of components of immune system. The number of neutrophils constitute as high as 90% of the total leukocyte population in the mammary gland during mammary gland infections. Neutrophils exert bactericidal effect through respiratory burst and further produces antibacterial peptides and defensins which kill wide range of mastitis causing pathogens (Sordillo and Streicher, 2002; Paape et al., 2002; Mehrzad et al., 2010).

1.4.1.2.2 Macrophages

Healthy and lactating mammary gland generally contains macrophages which can regulate both innate and acquired immunity. The nonspecific function of macrophages is to phagocytize bacteria and kill them with proteases and reactive oxygen species. However, the number of macrophages in the infected mammary

gland is lower possessing less Fc receptors leading to deceased rate of phagocytosis. The important function of macrophages in infected mammary gland is to recognize the pathogen and release pro-inflammatory cytokines which induce neutrophil recruitment to the site of infection. Besides, activated macrophages release prostaglandins, leukotrienes, and cytokines which can enhance local inflammatory response (Kampenand and Mallard, 1997; Kehrli et al., 1999).

1.4.1.2.3 Lymphocytes

If the invading pathogen survives the phagocytosis by neutrophils, the neutrophil infiltration is then replaced by T and B lymphocytes and monocytes (Wagstrom et al., 2000). Lymphocyte subset comprises of two main groups- T and B lymphocytes. T lymphocytes are responsible for cell mediated immunity and B lymphocytes are primarily responsible for humoral immunity. B lymphocytes are developed in bone marrow in adults. When a pathogen enters into the body of host, B lymphocytes uses specific receptors to connect to the antigen. Upon activation by T lymphocytes it proliferates into plasma and B memory cells. Plasma cells produce antibody specific to the antigen which is responsible to kill the antigen. Memory cells can remember the specific antigen and if the same antigen invades the host next time, it activates the immune system much faster than previous time and the antigen is destroyed.

T lymphocytes are produced in liver or bone marrow and later moved to the thymus mature. Similar to B lymphocytes, T lymphocytes also contain specific receptors which can recognize variety of antigens. T lymphocytes can be classified into two major groups: αβ T lymphocytes and γδ T lymphocytes. The αβ T lymphocytes include helper T cells (CD4+) and cytotoxic or regulatory T cells (CD8+). The primary function of T helper cells is to activate B lymphocytes and killer T cells. The killer T cells are expert in killing virus, bacteria and even cancer cells once it is activated. In healthy bovine mammary gland, the prevailing type of T lymphocytes is αβ T lymphocytes predominantly expressing CD8+ phenotype. However, during mastitis the predominant phenotype of αβ T lymphocytes is CD4+ (Asai et al., 1998; Wagstrom et al., 2000). γδ T lymphocytes have not been well characterised and their biological functions are not yet clear. However, limited data have suggested that this class of lymphocytes may act as cytotoxic and may contribute in defence mechanism of body against bacterial infections (Sordillo and Streicher, 2002).

Natural killer cells have cytotoxic activity similar to cytotoxic T cells. However, they do not have any T cell receptors to produce antibody, but can distinguish infected and cancerous cells from normal cells to destroy them. Research findings have indicated that natural killer cells can kill both gram positive and gram negative bacteria and thus highlights its importance in prevention of mastitis (Shafer-Weaver and Sordillo, 1997).

1.4.1.3 Soluble defence mechanisms

1.4.1.3.1 Antibodies

The primary soluble defence mechanisms in colostrum/milk are antibodies of different isotypes and cytokines released by macrophages. Antibodies in milk contribute the body's humoral to defense against pathogens in two different ways: by preventing entry of the pathogen by binding to specific epitopes, followed by removal of antigen-antibody complexes by macrophages and triggering killing of the pathogen by stimulating one or more complement pathways (Beck et al., 2010). Four different types of antibody isotypes which may be associated with the defence mechanism during mammary gland infections viz. IgG1, IgG2, IgM and IgA (Guidry and Miller, 1986). The highest level of immunoglobulins is recorded in the mammary gland secretion during the release of colostrum and during mammary gland inflammation due to infections. IgG1 and IgG2 are reported to present in high concentrations in normal and infected mammary gland secretions, respectively. The primary function of IgG1, IgG2 and IgM in the mammary gland is to increase phagocytosis by neutrophils (Mukkur and Inmam, 1989) and macrophages by acting as opsonins. On the other hand, secretory IgA is present in small quantities only raising doubts on its role in defence against mammary gland infections (Mukkur and Tewari, 1975). It causes agglutination of bacteria entered into the mammary gland to prevent colonization of bacteria and it does not act as opsonins.

1.4.1.3.2 Complement, lysozyme and lactoferrin

In addition to antibodies, the other important soluble defence mechanisms are complement, lysozyme and lactoferrin. Complement system is composed of several small proteins which are synthesized by liver and are present in milk and blood. The functions of complement are cell lysis, opsonization, clumping of antigen bearing

agents and attraction of macrophages and neutrophils for chemotaxis. Complement is the predominant proinflammatory mediator in mastitis caused by *E. coli* (Rainard and Poutrel, 1995; Riollet et al., 2000). Highest concentration of complement has neen reported during mastitis in contrast to the lowest concentration in healthy mammary gland during lactation (Rainard and Poutrel, 1995). Lysozyme and lactoperoxidase are the enzymes present in normal milk have bactericidal and bacteriostatic activities against both gram positive and gram negative bacteria, respectively. However, both enzymes are present in lower concentrations in bovine milk which limits their effectiveness in protection of bovine mastitis.

1.4.1.3.3 Cytokines

Cytokines acts as mediators as well as regulators of immune response (Belardelli and Ferrantini, 2002). The variety of cytokines reported to be present in healthy and infected mammary gland are interleukins (IL), colony stimulating factors (CSF), interferon gamma (IFN-γ) and tumour necrosis factor (TNF) [Alluwaimi, 2004]. The interleukins which are associated with bovine mammary gland infections are IL1, IL2, IL6 and IL8 (Alluwaimi, 2004). Of all the interleukins associated with bovine mastitis, IL2 is most extensively studied and characterized (Alluwaimi, 2004). IL2 is produced by T lymphocytes during immune response. IL2 is necessary for maturation of regulatory T cells and development of T lymphocytes immunologic memory. There are reports that administration of IL2 via intramammary route can enhance both cell mediated and humoral immune response in *S. aureus* infected quarters (Nickerson et al., 1989). Administration of IL2 therapeutically by intramammary route can protect infection of mammary gland against *S. aureus* infection. However, it failed do so in case of already existing infections of mammary gland in cows (Daley et al., 1991). Due to the influence of colony stimulating factors (CSF) on phagocytic cell populations, various researchers suggest their importance in clinical applications to treat bovine mastitis (Daley, et al., 1993). Interferon gamma (IFN-γ) plays a critical role in both innate and acquired immunity against bacterial and viral infections by activating the macrophages may play an important tool in the prevention and control of bovine mastitis (Sordillo and Babiuk, 1991). Tumor necrosis factor (TNF) is a member of group of cytokines which is primarily produced by activated macrophages and can also be produced by NK cells and CD4+ lymphocytes. This factor regulates immune cells by taking active part in neutrophil

recruitment. In acute mastitis caused by *E. coli*, TNF-α was reported to be the predominant cytokine during early stage of infection (Slebodzinski et al., 2002). If the production of TNF-α could be modulated during coliform mastitis, there is a fair chance to reduce the infection rate of bovine mastitis caused by *E. coli*.

1.5 Current status of different strategies used for therapy and prevention of bovine mastitis

1.5.1 Antibiotics

Antibiotics ranging from narrow to broad have been used extensively over the past 40 years in the control of bovine mastitis (Barkema et al., 2006). However, because of the emerging antibiotic resistance believed to be probably due to their overuse (Pearson, 2002; Shi et al., 2010; Shittu et al., 2011; Park et al., 2012) and the induction of prolonged persistent antibiotic resistance in biofilms by many mastitis-causing pathogens, as demonstrated recently for *S. aureus* isolated from cases of bovine mastitis in the bovine mammary gland (Babra et al., 2013), effectiveness of antibiotic therapy has been compromised. As such the control of bovine mastitis has become one of the most challenging problems on dairy farms today. Cows suffering from mastitis are culled due to high SCCs and repeated occurrence of clinical mastitis. Although culling and selective antibiotic therapy have been found to cause a reduction in the manifestation of clinical mastitis (Petersson-Wolfe et al., 2010), dairy farmers are often reluctant to cull affected cows due to the devastating financial impact on backyard farmers due to losses in milk production as is often the case in developing countries.

Intra-mammary infections have been traditionally treated with systemic or intra-mammary antibiotic therapy (Barlow, 2011). Despite scheduling treatment regimens to prolong the availability of appropriate antibiotics for an extended period of time in the infected area (Erskine, 2001), the cure rates of mastitis particularly for *S. aureus* infections have been reported to vary from 0% to 80% (Erskine, 2001; Timms, 2001; Gruet et al., 2001). This is presumably due to the induction and persistence of biofilm-associated antibiotic resistance (Babra et al., 2013) depending upon the intensity of infection as reflected by SCC counts and management practices (Serieys et al., 2005; Petrovski, 2007). Milking animals with a SCC of less than 1 million

showed greatest success of antibiotic therapy (Cattell and Belschner, 1997) while those with a higher count responded poorly (Timms, 2001). This is notwithstanding the fact that some antibiotics including penicillin, oxytetracycline, lincomycin and neomycin may affect the phagocytic properties of polymorphonuclear leukocytes (PMN) by altering the oxidative burst property of PMN (Paape et al., 2003) leading to a recurrence of intra-mammary infections.

1.5.2 Bacteriophage therapy for mastitis associated infections

Given the problems associated with antibiotic therapy of mastitis, development of alternative treatment strategies for management of clinical and sub-clinical mastitis are warranted. One such alternative treatment is bacteriophage therapy, which uses pathogen specific bacteriophages in the treatment of a bacterial infection. Recent interest in phage therapy in veterinary medicine was sparked by some early success in the treatment of *E. coli* infections in animal models including a chicken model for respiratory infections (Huff et al., 2002), a mouse model for meningitis (Smith and Huggins, 1982) and a calf model for diarrhoea (Smith and Huggins, 1987). However, the few studies that have been carried out using bacteriophages to treat mastitis caused by *S aureus* infection have yielded variable results. While intra-mammary infusion of bacteriophage into *S aureus* infected quarters of lactating dairy cattle did not show significant protection (Gill et al., 2006 a, b). Kwiatek and co-workers in 2011 isolated and characterised a bacteriophage from the milk of cows suffering from mastitis with broad-spectrum activity against methicillin- resistant *S aureus* (MRSA). It is suggested that additional research is required to explore the therapeutic potential of bacteriophages to treat clinical and subclinical mastitis associated bacterial infections.

1.5.3 Genetic selection of cattle for resistance to clinical mastitis

Breeding production animals for resistance to infectious diseases is not new (Adams and Templeton, 1998), but generally the breeding of farm animals has been confined to enhancement of production traits such as increased milk production. Such selection, while enhancing milk production, has been reported to increase the incidence of many infectious diseases including bovine mastitis (Simianer et al., 1991; Heringstad et al., 2007). In attempts to overcome this problem, a strategy based on enhancing the overall immune response including antibody-mediated

immune response (AMIR) and cell-mediated immune response (CMIR) has been proposed (Thompson-Crispi et al., 2012). However, a negative genetic correlation between AMIR and CMIR (Heriazon et al., 2009) has been recorded, making a balanced genetic selection more complicated and so requiring further investigation.

1.5.4 Ancillary non-specific strategies for prevention of mastitis

1.5.4.1 Non-specific immunostimulants against mastitis

In the absence of the availability of effective commercial vaccines for prevention of mastitis caused by multiple pathogens, attempts are continually being made to evaluate the potential of non-specific immunostimulants for prevention of bovine mastitis (Eid et al., 1995)**.** Lysate of *Corynebacterium cutis* has been considered as one of the non-specific immune stimulants against mastitis. There are reports of a reduction in SCC (Lee et al., 1996) in the milk of dairy cows receiving a subcutaneous injection of lysate of *C. cutis.* Intramuscular injection of *C. cutis* to pregnant ewes resulted in an increased level of IgG in serum on the 140th day and in colostrum up to 3 days post-parturition (Yilmaz et al., 2011). Clearly, further studies on the potential of the non-specific immunostimulants are warranted.

1.5.4.2 Lactation therapy

Treatment of mastitis during lactation with antibiotics is referred to as "lactation therapy", which is used by many producers to reduce the clinical signs of mastitis and bring back the normal milk production of cows. This therapy has proven useful in reducing the SCC in milk and thereby maintains the quality of milk (Oliver et al., 2004; Pyorala, 2009). However, lactation therapy for subclinical mastitis is not suggested as it is not economically viable and shows poor efficacy (Pyorala, 2009). Factors such as SCC in milk during treatment, stage of lactation, immune status of the animal, age of the cow and type of pathogen also play an important role in the success or failure of lactation therapy (Hillerton and Berry, 2003; Hektoen et al., 2004). New intra-mammary infections in cows have been shown to respond better to antibiotic therapy than chronic infections (Pinzon-Sanchez et al., 2010), and young animals show better response to treatment than older animals (Sol et al., 2000; McDougall et al., 2007a, b). Spontaneous cure by lactation therapy for clinical as well as subclinical mastitis caused by *S. aureus* is very rare (Oliver et al., 2004; Zhen et al., 2009). Lactation therapy in chronic clinical cases of mastitis caused by *S.*

aureus has been found equally ineffective (Petersson-Wolfe et al., 2010). However, an extended period of treatment with antibiotics at therapeutic levels has been reported to yield better cure rates for clinical mastitis caused by *S. aureus* (Sol et al., 2000). A serious drawback of this therapy is the loss of milk because of antibiotic residues.

1.5.4.3 Dry cow therapy

Dry cow therapy with antibiotics has been suggested as one of the options to control intra-mammary infections and prevent development of mastitis (Oliver and Sordillo, 1988). During the dry period, the cow is at the greatest risk of acquiring new intra-mammary infections with both gram-positive and gram-negative environmental or contagious pathogens (Todhunter et al., 1991). It has been reported that about 61% of new infections are acquired during this period (Todhunter et al., 1991). Treatment during dry period is advantageous because it allows treatment of infections with antibiotics without the need to discard milk from treated quarters. Antibiotics are administered towards the end of lactation (Janosi and Huszenicza, 2001) and may remain in the udder in concentrations high enough to kill pathogenic bacteria for 20-70 days, depending upon the kind of formulations that are used. The antibiotic has an enhanced penetration due to prolonged exposure and the probability of curing intra-mammary infections increases markedly, unless resistance to new antibiotics is acquired by the invading pathogen's biofilm formation in the udder (Babra et al., 2013). Dry cow therapy has been reported to eliminate almost 100% of mastitis caused by *S. agalactiae* (Oliver and Sordillo, 1988). However, dry cow therapy is comparatively less successful to prevent *S. aureus* mastitis than streptococcal mastitis (Ziv et al., 1981). The cure rate of dry cow treatment against *S. aureus* mastitis was reported as approximately 50% and vaccination against this pathogen during the dry period may enhance the antibiotic efficacy (Todhunter et al., 1991). Dry cow therapy for a period of two weeks showed significant reduction in the number of clinical mastitis cases due to infection with *S. dysgalactiae* and *S. uberis* (Hassan et al., 1999).

1.5.4.5 Teat sealer

The development of internal and external teat sealants for use during the dry period is a promising progress towards control of mastitis and its aftermath (Twomey et al.,

2000; Sanford et al., 2004; Ruciman et al., 2010; Izak et al., 2012). External teat sealants such as DryFlex and Delaval also showed potential in reducing new infections of the mammary gland during the dry period (Timms, 2001). However, lack of persistence is the main drawback of external teat sealers (Hayton and Bradley, 2001). Bismuth subnitrate as an internal teat sealer used in field conditions was reported to reduce new infections up to ten fold (Huxley et al., 2002). Internal teat sealer used with long acting antibiotics during the dry period showed a 30% and 33% reduction in new intra-mammary infections and incidence of clinical mastitis, respectively (Sordillo et al., 1997). Bismuth subnitrate combined with cloxacillin as dry cow therapy demonstrated reduction in both clinical and subclinical cases of mastitis (Ruciman et al., 2010; Izak et al., 2012).There are several studies that have demonstrated the usefulness of OrbeSeal, an internal sealer in reducing new infections of the mammary gland in lactating animals (Cook et al., 2004; Sanford et al., 2004).

1.6 Bovine mastitis in Australia

The Dairy industry is the third largest rural industry in Australia after beef and wheat industry providing direct employment in the dairy farms to approximately 43,000 people. The national dairy herd is composed of 1.65 million cows producing 9,200 million litres of milk. Australian Dairy industry in is the major export industry generating a total of $2.76 billion per annum and contributing 7% to the World Dairy Trade (Dairy Australia, 2013). Dairying is the prominent industry in the temperate and sub-tropical zones of Australia. All states of Australia produce and supply fresh milk and milk-products including custard, cheese and yoghurt to the nearby towns and cities. However, the south-east seaboard states produce the highest amount of milk in Australia.

Mastitis continues to be one of the most economically devastating diseases of dairy cattle in Australia. More than $130 million is lost by the Australian dairy farmers ($A200/cow/year) every year due to poor udder health caused by mastitis resulting in reduction of milk production, increase in treatment costs, veterinary consultation fees, number of cow culls (Dairy Australia, 2011). A herd without an effective mastitis control programme may witness morbidity as high as 40% with infection, on

an average of two quarters of the mammary gland (Dairy Australia, 2011). Of the various clinical manifestations, subclinical mastitis is economically the most important due to its long term effects on milk yields and needs specific tests for detection (Gogoi, 1997; Zafalon et al., 2007).

It is difficult to establish the true incidence of mastitis in Australia as there are variations in geographical locations, type of farming, management and herd size in different states of the country. A survey in one district cannot represent another district (Frost et al., 1966). Moreover, incidence of mastitis and cell counts in milk has been reported to be higher in the flood hit areas and during rainy season. It is even complicated due to subclinical mastitis which is often invisible and un-noticed. The trend of aetiology of mastitis has been variable in Australia. Since 1960 to 1990 the predominant causative agent of mastitis was *S. aureus* and *S. agalactiae*. The less common mastitis pathogens were *S. dysgalactiae* and *S. uberis*. According to a report of the Australian Veterinary Journal most herds were infected with *S. aureus*. However, there was variation of infected cows among the infected herds. A total of 100% of herds in Brisbane Milk Supply area were infected by *S. aureus* comprising 46% infected cows. In New South Wales the percentage of herd infection was lower in Camden and Laidley but it was as high as 86% in Moss Vale District. The incidence of *S. agalactiae* infection was 86% in herds including 29.7% infection in cows, 90.7% herd infection in Moss Vale, 15% in Camden and Laidley, respectively. Western Australia has as low as approximately 2% cows infected with *S. agalactiae* (Frost et al., 1966).

In the last 10-20 years there has been a change in the trend of occurrence of bovine mastitis in Australia. Environment pathogens have become more prominent causative agents of bovine mastitis in most herds throughout the country. *S. uberis* has topped the list of environmental pathogens causing mastitis followed *by Pseudomonas*, *Bacillus cereus* and *E. coli* (Mein et al., 2011). A recent study on the prevalence of mastitis causing organisms in intensive dairy herds in New South Wales revealed a shocking 91% of environmental pathogens which included 41.6% environmental S*treptococcus and* 33.6% coliforms (Shum et al., 2009). *S. uberis* was the predominant environmental *Streptococcus* followed by *Strep. dysgalactiae* and *E. coli* being the most common coliform in those dairy herds. However, prevalence of

the contagious pathogens, *S. aureus* and *S. agalactiae*, still remains an unsolved battle in many dairy herds in Australia, *S. aureus* being the predominant causative agent of clinical bovine mastitis. Moreover, overuse or misuse of antibiotics to treat *S. aureus* mastitis has led to the development of a new genre of *S. aureus* which is multi-drug resistant including Methicillin called MRSA (Methicillin Resistant *Staphylococcus aureus*). Given the recent reports that even MSSA (Methicillin Sensitive *Staphylococcus aureus*) can become resistant to certain antimicrobials when presented as biofilms adds another dimension to bovine mastitis caused even by MSSA (Babra et al., 2013).

The first widely adopted Mastitis Control Program in Australia was "Five Point Plan" consisting the original five points to reduce the number of prevalent and new mastitis infections. The plan lasted for 30 successful years and after 30 years it became less viable and less cost-effective in pasture based dairy herds and the plan failed to control the prevalence of mastitis caused by environmental pathogens. After "Five Point Plan", important step implemented towards the control of mastitis in Australia was "Farm Guidelines for Mastitis Control" which was developed by Countdown Downunder technical group and first published in 1998 by Dairy Research and Development Corporation. Every effort was made to distribute the guidelines to dairy farmers. This programme has been a success in Australia with its associated problems with implementation. The current management practices followed to control mastitis in Australia are aimed at reducing the level of herd infection, achieving a bulk milk cell count (BMCC) below 400,000 cells/ml, improving quality of milk in terms of its composition, improving udder health and recognizing and controlling pain and inflammation caused by mastitis.

1.7 Significance of *Staphylococcus aureus* in bovine mastitis

Staphylococci in human pus were described for the first time by Robert Koch in 1878 and the name "Staphylococcus" was first suggested by Alexander Ogston in 1884. Two different coloured colonies namely yellow and white produced by Staphylococci were first described by Rosenbach in 1884 suggesting the nomenclatures *S. aureus* and *S. albus* which was later renamed as *S. epidermidis*.

Staphylococci are gram positive non-motile, non-spore forming facultative anaerobes. They are about 1µm in diameter cocci and form grape like clusters.

Human is the main reservoir of *S. aureus* organisms (Banwart, 1979). As these organisms are present on hands, skin, anterior nares, infected wound, pimples, nose and throat discharges of human, the health, hygiene and work habit of milkers, animal attendants can influence the level of contamination of milk. The persistent and intermittent colonization of *S. aureus* in anterior nares of healthy people are reported to be as high as 20% and 70%, respectively (Kluytmans et al., 1997) which may lead to staphylococcal infections (Saginur et al., 1996). In healthy dairy animals *S. aureus* is present in almost all external surfaces (Roberson et al., 1998) which can multiply and persist for short period (Kloos, 1997). However, udder has been the most preferred site of *S. aureus* colonization in dairy animals (Jorgensen et al., 2005) and transmission usually takes place during milking (Roberson et al., 1994). There are also reports of *S. aureus* surviving in feed material, dust, bedding which can serve as a source of infection (Matos et al., 1991).

Despite advances in the area of prevention and control of mastitis, *S. aureus* is the leading cause of contagious bovine mastitis worldwide (Athar, 2007; Dingwell et al., 2003; Keefe et al, 1997). It causes contagious mastitis resulting either clinical or subclinical mastitis with increase in the number of somatic cell count (SCC) in milk. It has been reported to cause more than 80% of subclinical mastitis cases in bovines causing a loss of US $ 300 per animal per year (Wilson et al., 1997; Karahan et al., 2011). *S. aureus* enters the mammary gland via teat orifice. Chapped or injured teats are most likely to be affected. Once the organism enters into the mammary gland, it adheres to epithelial lining and invades the host immune system by variety of antiphagocytic factors including capsule, proteinA, fibronecting binding proteins. Once intra-mammary infection is established, damage to the mammary gland epithelial lining is initiated by ulceration and occlusion of lactiferous ducts and alveoli, infiltration of inflammatory cells in the parenchyma. Mammary tissue damage is further complicated by various toxins produced by *S. aureus* which act as super antigens.

The success rate of treatment against *S. aureus* mastitis is rare and it relapses in most of the cases (Barkema et al., 2006). There is heavy reliance on the use of antibiotics to control bovine mastitis as there are no prophylactic accredited vaccines against the malady nor are there any preventive measures which are fully dependable. As such there is an abundance of literature concerning susceptibility of mastitis pathogens against a large spectrum of antimicrobial agents. However, worldwide changes in resistance patterns to antimicrobial agents of *S. aureus* strains isolated from clinical sites have been observed (Ross et al, 1974; Eykyn, 1988). Misuse or overuse of antimicrobial agents and introduction of resistant strains into countries have been responsible for an increase in the prevalence of strains resistant to antibiotics (Ross et al, 1974; Gogoi and Dutta, 2001) including methicillin, a penicillinase resistant antibiotic, the first case of MRSA being reported from England (Jevons, 1961). Within the past decade MRSA has emerged as a problematic pathogen in hospitals as well as in the dairy industry. As a result, vancomycin is currently being used effectively against MRSA, the first case of vancomycin resistance being reported by Pearson (2002). Given the recent reports that even MSSA can become resistant to certain antimicrobials when presented as biofilms adds another dimension to bovine mastitis caused even by MSSA (Babra et al., 2013).

1.7.1 Virulence factors of *S. aureus*

S. aureus produces a variety of virulence factors by which it causes damage to host leading to signs and symptoms of disease. These virulence factors permit the pathogen to evade host immune mechanisms by adhering to eukaryotic membrane, resisting phagocytosis and killing eukaryotic cells. Broadly, the virulence factors of *S. aureus* can be classified into two groups-(a) Virulence factors involved in establishment of infection and (b) Virulence factors involved in maintenance of infection (Haveri et al., 2007).

1.7.1.1 *Staphylococcus aureus* virulence factors involve in establishment of infection

The first step in establishing infection is the initial attachment of *S. aureus* to eukaryotic membrane followed by colonization and subsequent infection (Brouillette et al., 2003). Colonization is commonly associated with a variety of adherence

factors or adhesins which are known as microbial surface component recognizing adhesive matrix molecules (MSCRAMMs).These surface molecules attach to components of host tissue or plasma (Gordon & Lowy, 2008; Bien, et al., 2011). There are over 20 different MSCRAMMs identified which can be expressed in *S. aureus* (Walsh, et al., 2008) and they mediate attachment to surface proteins of host cells including collagen, elastin, fibrinogen, thrombospondin, fibronectin, bone sialoprotein and laminin (Bartlett and Hulten, 2010). Major adhesins in this group that mediate the initial attachment of bacteria to the bovine mammary gland, providing the first critical step for establishing infection (Foster and Hook, 1998) are *S. aureus* Protein A, clumping factors A and B (ClfA and ClfB), protein A, Collagen adhesion (CNA) and the fibronectin binding proteins A and B (FnBPA and FnBPB) [Cucarella et al., 2001; Gordon and Lowy, 2008; Plata et al., 2009]

1.7.1.1.1 Protein A (Spa)

Protein A is one of many surface proteins produced by *S. aureus* which has been considered to have antiphagocytic properties based on its ability to bind the Fc portion of immunoglobulin G (IgG). It consists of a single polypeptide chain of molecular weight 42,000 and contains little or no carbohydrate (Bjork, 1972; Sjoquist, 1972). It has the ability to interact with several host components, possibly indicating a role as a virulence factor in *S. aureus* infections. More than 7200 different spa types have been reported due to the variable number of repeats in spa region (http://spaserver.ridom.de/).The virulence factor of Spa in *S. aureus* has been proved in murine septic arthritis (Palmqvist et al., 2002).

1.7.1.1.2 Clumping factors /Fibrinogen binding proteins (Clf)

Clumping factor (ClfA and ClfB), a fibrinogen-binding protein anchored to the *S. aureus* cell wall, is a virulence factor contributing to murine septic arthritis (Josefsson, 2001) in mice and rat endocarditis (Moreillon et al., 1995). The fibrinogen molecule contains two sites available which interact with clumping factor. ClfA binds to C terminus of γ-chain (Hartford et al., 1997; Hawiger et al., 1982; McDevitt et al., 1997) and ClfB binds to α-chain (Walsh et al., 2008). The characteristic clumping of *S. aureus* cells in fibrinogen-containing medium is mediated by this binding interaction. It has been shown that ClfA through its fibrinogen-binding function is a mediator of *S. aureus* -induced platelet aggregation

(Bayer et al., 1995; O'Brien et al., 2002). ClfB is one of the determinants of nasal persistence of *S. aureus*. Mice immunized with recombinant ClfB via intranasal route have demonstrated reduction of colonisation and persistence of *S. aureus* in nares of mice (Schaffer et al., 2006).

1.7.1.1.3 Fibronectin binding proteins (FnBP)

Fibronectin (Fn) is a 440-kDa glycoprotein found in the extracellular matrix and body fluids of animals. It was the first ECM (Extracellular matrix protein) shown to act as substrate for the adhesion of eukaryotic cells (Hynes, 1990). The fibronectin-binding proteins A and B (FnBP-A and FnBP-B), encoded by the *fnbpA* and *fnbpB* loci, respectively, were identified as the main adhesins that bind purified fibronectin (Jonsson et al., 1991; Signas et al., 1989). At least one of the FnBP genes is found in the vast majority of *S. aureus* strains (Smeltzer et al., 1997). Pathogen adherence to the teat canal of the udder is believed to be the first step of mammary gland infection, and FnBPs have been proposed as virulence factors in bovine mastitis (Sutra and Poutrel, 1994). Mamo et al., (1994) reported that mice immunized with FnBP were partially protected against intra-mammary challenge.

1.7.1.2 Virulence factors involve in maintenance of infection

S. aureus produces a variety of virulence factors which evade the tissue and host immune system and thereby maintain infection. These virulence factors are capsular polysaccharides, enzymes, cytotoxins, superantigens.

1.7.1.2.1 Capsular polysaccharides

The capsular polysaccharide or capsule is a cell wall bacterial component that protects bacteria from phagocytic uptake and enhances microbial virulence. *S. aureus* isolated from human infections have been shown to produce 12 polysaccharide serotypes (11 different capsular serotypes) and one surface polysaccharide 336 (Guidry et Al., 1998). The majority of clinical isolates were reported to express a surface polysaccharide of either serotype 5 or 8, representing 70-80% of the isolates from all sources (Arbeit et al., 1984; Hochkeppel et al., 1987; Poutrel et al., 1988). However, the percentage distribution of these capsular types appears to vary in different countries (Arbeit et al., 1984; Hochkeppel et al, 1987; Poutrel et al, 1988; Roghmann et a.l, 2005; Han et al, 2000; Sompolinsky et al, 1985). More recently, it

has been suggested that there are only four types of CPs (Lee, 2002), all other reported types representing mutated versions, details of which has not been elucidated. CPSs from serotypes 1, 2, 5, and 8 have been purified and characterized (Fattom et al., 1996; Liau and Hash 1977; Murthy et al., 1983; O'Riordan and Lee, 2004; Jones, 2005). Purified capsular polysaccharides are non-immunogenic in mice (Fattom et al, 1990) but which become immunogenic upon linking to carrier proteins to produce and induce T-cell dependent immune responses.

1.7.1.2.2 Virulence-associated enzymes

Most *S. aureus* strains express Staphylokinase which acts as plasminogen activator (Gogoi Tiwari and Tiwari, 2006; Kwiecinski et al., 2010) Staphylokinase forms a complex with plasminogen which activates proteolytic activity leading to dissolving of fibrin clots. Other enzymes produce by *S. aureus* are lipase to digest lipids, deoxyribonuclease (DNAse) to break down DNA, coagulase to clot plasma, hyaluronidase to break down hyaluronic acid, beta lactamase for penicillin resistance and fatty acid modifying enzyme (FAME) to provide nutrient to *S. aureus* and anti-bacterial lipids (Cheesbrough, 1995).

1.7.1.2.3 Cytotoxins

A large number of cytotoxins are produced by *S. aureus* which form pores in the cell membrane causing osmotic swelling leading to cell death. These cytotoxins include leukocidins, phenol soluble modulins (PSMs) and cytolysins. The cytolysins of *S. aureus* are α-, β-, γ-, and δ-toxins, of which α-toxin is well characterised and most potent toxin (Wadstrom 1983). β-toxin is a sphingomyelinase C and 95% of *S. aureus* isolates from bovine mastitis cases produce this toxin (Tollersrud, 2001) which causes damage to epithelial lining of mammary gland. γ-toxin is produced by coagulase positive *S. aureus* causes tissue damage by stimulating degranulation of neutrophils. Delta toxin is responsible for lysis of cells in different species (Hirsh et al., 2004). Leukocidins target the phagocytic cells and cause pore in the membrane of PMNs and interfere with the function of neutrophis (Rainard et al., 2003). The members of Leukocidin family are Panton Valentine Leukocidin (PVL), LukR-PV, LukM/FPV, LukE/D, γ-haemolysin and Leukocidin**.** Phenol soluble modulins (PSMs) are the peptides produced by *S. aureus* which are cytotoxic and

proinflammatory (Surewaard et al., 2012). Recent finding has demonstrated that it plays a part in the formation of *S. aureus* biofilm (Periasamy et al., 2012).

1.7.1.2.4 Superantigens

S. aureus produces a number of superantigens including enterotoxins (SEs), Toxic Shock Syndrome toxin and exfoliative toxins. Enterotoxins of *S. aureus* include the classical enterotoxins A to E and the recently identified and characterised SEG-SEU toxins (Dinges et al., 2000; Smyth et al., 2005). These antigens are considered superantigens due to their ability to induce release of cytokines from both T cells and macrophages by binding to outer surface of MHC class II proteins and T cell receptors (Marrack and Kappler, 1990; Balaban and Rasooly, 2000; Ulrich, 2000; Schlievert, 2008).

1.7.2 Biofilm formation by *S. aureus* and bovine mastitis

1.7.2.1 Biofilm

Bacterial growth can be classified into two types: single cells and aggregated cells. Single cell growth is known as planktonic and the aggregated cells are known as biofilm. Definition of biofilm according to Bjarnsholt, 2013 is "A coherent cluster of bacterial cells embedded in a matrix, which is more tolerant of most antimicrobials and host defences compared with planktonic bacterial cells". The first ever publication on biofilm was published by Hoiby in 1977 who described aggregates of *Pseudomonas aeruginosa* in lungs of cystic fibrosis patients. However, the term "Biofilm" was first used in 1981 by Costerton et al. to describe bacteria embedded in extracellular matrix (McCoy, 1981). Since then there has been vast research on biofilms formed by different bacteria (Nickel et al., 1989; Post et al., 1996; Erdos et al., 2003; Stoodley et al., 2005; Hiller et al., 2007, Babra et al., 2013 and Bjarnsholt, 2013) describing its potential role in the establishment of 80% of infections (Davies, 2003).

1.7.2.2 Implication of biofilm in various infections

Complexity and severity of various human and animal infections are contributed by biofilm formation by the respective causative organism. Biofilms of Staphylococcal and Streptococcal species complicates endocarditits, biofilms of *S. aureus*,

Haemophilus influenza and *Pseudomonas aeruginosa* complicates cystic fibrosis, *S. aureus* and *S. epidermidis* cause complications in implant related infections (Hall-Stoodley et al., 2004). These complications arise due to tolerance to phagocytosis (Matz et al., 2004; Matz et al., 2005; Bjarnsholt, 2013) and decreased susceptibility to antimicrobial agents (Bjarnsholt, 2013). Quorum sensing demonstrated by biofilms (Manefeld and Turner, 2002) also contributes to pathogenesis of disease. Quorum sensing is a biofilm dependant trait which involves cell to cell communication (Fuqua et al., 1994) and detachment of biofilm lead to septicaemia and colonisation.

1.7.2.3 Development of biofilm by *S. aureus*

S. aureus biofilms are considered major facilitators of different animal and human infections contributing 80% of all infections (National Nosocomial Infections Surveillance, 1999). Development of biofilm by *S. aureus* occurs in three steps. First step involves adherence of *S. aureus* cells to surface by physicochemical interactions. Various adhesion proteins of *S. aureus* including FnBPA, FnBPB, ClfA, ClfB, CNA and Bone sialo binding protein (BBP) play role in the adherence of *S. aureus* to extracellular matrix of host tissue (Patti et al., 1992; Mc Devitt et al., 1994; Tung et al., 2000 and O'Neill et al., 2008). The second step involves multiplication of *S. aureus* and cell to cell interaction and leading to formation of slimy layer like structure. The major component of *S. aureus* biofilms is an exopolysaccharide, Poly-β-1, 6-linked *N*-acetylglucosamine (PNAG) [Sadovskaya et al., 2007]. Four proteins including IcaA, IcaD, IcaB and IcaC encoded by the icaADBC operon are associated with the production of PNAG. IcaA and IcaD are the most important proteins for the production of PNAG (Otto, 2009). Besides PNAG, some other proteins of *S. aureus* have been described to be involved in cell to cell interaction and biofilm formation. These proteins include Bap, ProteinA, Agr, SasC and SasG (Lasa and Penades, 2006; Corrigan et al., 2007; Merino et al., 2009; Schroeder et al., 2009; Boles and Horswill 2011). Extracellular DNA (eDNA) has also been demonstrated to play an important role in biofilm formation during the attachment stage of biofilm (Archer et al., 2011). The third step involves detachment of biofilm. An accessory gene *agr* encoding agrA, agrB, agrC and agrD is responsible for detachment of biofilm helping in dissemination *S. aureus* resulting in chronic mastitis. The quorum sensing of *S. aureus* during biofilm formation is regulated by *agr* gene (Archer et al., 2011; Boles

and Horswill, 2011; Otto, 2013). It upregulates the enzyme responsible for degrading components of extracellular matrix and down regulates adhesive protein genes which maintain attachment of biofilm (Boles and Horswill, 2011; Otto, 2013), thus facilitating detachment of biofilm. It is also responsible for transient upregulation of certain genes which are responsible for production of toxins such as δ-toxin, capsule and protease (Boles and Horswill, 2008) thus allowing biofilm to neutralise kill neutrophils (Anwar et al., 2009).

1.7.2.4 Biofilm and *S. aureus* bovine mastitis

After entry into the mammary gland, *S. aureus* attaches to mammary gland epithelia and forms colonies which are surrounded by extracellular matrix leading to biofilm formation. Chronic persistence of infection is characteristic of biofilm related infection which is promoted due to resistance to phagocytosis and antibiotic therapy conferred by biofilm formation by *S. aureus* (Monzon et al., 2002).

Numerous investigators have demonstrated the ability of *S. aureus* to form biofilm *in vitro* by using various methods including Congo red agar plates and tissue culture method (Vasudevan et al., 2003; Oliveira et al., 2007; Dhanawade et al., 2010; Babra et al., 2013). However, there are also demonstrations of biofilm formation by *S. aureus* in the mammary gland (Perez et al., 2009; Prenafeta et al., 2010). Production of extracellular matrix was demonstrated by Watson et al. as early as 1989 by using electron microscopy. They termed it as pseudo capsule which was observed in *S. aureus* isolated from clinical mastitis cases in cows and ewes. In vivo production of extracellular matrix by *S. aureus* was demonstrated indirectly by establishing production of antibodies against PNAG (Perez et al., 2009) and slime associated antigenic complex (SAAC) [Prenafeta et al., 2010] in experimental production of mastitis in sheep and cows.

1.8 Current status of vaccine development against bovine mastitis

Mastitis is caused by variety of pathogens and as such various efforts have been made to develop vaccine against mastitis caused by various organisms. It is beyond the scope of this review to describe detailed experimental approaches used in the

development of vaccines against bovine mastitis caused by the major bacterial pathogens. As such, a brief description of the vaccines, including the approaches currently used in practice or promising prototype vaccine candidates, is presented. The use of vaccination particularly with autogenous killed whole cell vaccines to control infectious diseases on-farm in dairy cattle is common, and vaccination against mastitis pathogens is no exception. Several efforts have been made to develop a vaccine against mastitis, but few have claimed satisfactory outcomes (Lee et al., 1988; Mamo et al., 1994; Leitner et al., 2000 and 2003; Tenhagen et al., 2001; Nour El-Din et al., 2006; Pellegrino et al., 2010; Xu et al., 2011) in the field or backyard farms. It is clear that a single vaccine will not prevent mastitis caused by the plethora of pathogens and their different mechanisms of pathogenesis (Health, 2011).

1.8.1 Vaccines against coliform bacteria

Coliforms (*E. coli, Klebsiella sp.*) are etiological agents of environmental mastitis. Coliform mastitis generally causes clinical mastitis mostly during the peri-parturient period (Hogan and Smith, 1987; Jones 2009b). Early investigations used heterogeneous oligosaccharide antigens derived from *E. coli* to develop a vaccine against coliform mastitis. These vaccines were administered during the non-lactating period with the aim of preventing mastitis in subsequent lactations (Wilson 1972; Wilson et al., 1972). These vaccines reduced the severity of infection initially, but their effect gradually diminished over time (Dosogne et al., 2002). J-5 Bacterin, Mastiguard™ and J Vac® are the three vaccines available in the market against coliform mastitis (Ruegg, 2005). Startvac (Hipra) has been made available in the market targeting not only coliforms but also coagulase-negative staphylococci and *S. aureus.* J-5 Bacterin also known as the *E. coli* J5 vaccine is composed of the J5 mutant strain of *E. coli.* Cows vaccinated with this vaccine showed a significant reduction of clinical mastitis cases under field conditions (Gonzalez et al., 1989; Hogan et al., 1992). Only 20% of the vaccinated animals showed clinical infections, although there was no difference in the incidence of new coliform mastitis cases among the vaccinated compared to the non-vaccinated animals (Hogan et al., 1992). However, no vaccines against mastitis caused by *K. pneumoniae* are available in the marketplace.

1.8.2 Vaccines against *Streptococcus uberis*

The high global incidence of clinical mastitis due to *S. uberis*, an environmental pathogen, has warranted the development of vaccines to prevent mastitis caused by this specific etiological agent (Leigh, 2000). Repeated immunisation in experimental animals with killed *S. uberis* vaccine resulted in a significant reduction in the number of bacteria in milk but failed to reduce the SCC count (Finch et al., 1994). Vaccination with this bacterin was reported to offer protection against mastitis caused by the homologous *S. uberis* strain but failed to protect against a heterologous strain (Finch et al., 1997). In another study, plasminogen activator derived from *S. uberis* showed promising results in reducing the severity of infection (Leigh, 1999 and 2000). In a recent study, cows vaccinated with a recombinant adhesion molecule of *S. uberis (*rSUAM) by the subcutaneous route showed an increased antibody titre in milk and serum, which was found to reduce adherence and internalisation of the organism into the epithelial cells of the mammary gland under *in vitro* conditions (Prado et al., 2011).

Numerous efforts to develop *S. uberis* vaccines to prevent mastitis have not proven successful and no commercial vaccines for prevention of this infection are available in the market. No immune response is induced in the mammary gland even after intra-mammary infection with *S. uberis*, which further complicates the development of a vaccine (Schukken et al., 2009). Sortase-anchored proteins derived from *S. uberis* may be potential candidates for vaccine as they are potential virulence antigens contributing to the pathogenesis of bovine mastitis (Leigh et al., 2010). Recently, Denis et al., 2011 reported that cows suffering mastitis after environmental exposure to *S. uberis* developed bactericidal antibodies and T cells in blood and milk. This resulted in an increased level of interferon-gamma (IFN-γ) that was specific for *in-vitro* killing of *S uberis* (Denis et al., 2011). Clearly, research on the development of an effective vaccine against *S. uberis*-associated mastitis is highly warranted.

1.8.3 Vaccines against *Streptococcus agalactiae* and *Streptococcus dysgalactiae*

S. agalactiae is an important pathogen for humans (infants, pregnant women and immunocompromised elderly patients) has nine serotypes (Health, 2011), each

having a serologically distinct polysaccharide capsule; a capsular conjugate vaccine, using the capsule of predominant serotypes, has been evaluated in Phase 2 trials with encouraging results. However, this pathogen causes mastitis in cattle with little information available on the capsular types prevalent in the dairy population. Furthermore, no commercially attractive prototype vaccine candidates are available against mastitis caused by *S. agalactiae* or *S. dysgalactiae* despite the fact that many attempts have been made to develop an effective vaccine against these pathogens (Keefe, 1997; Gillen 2007). Recently, a recombinant vaccine composed of *S. aureus* cluming factor A (ClfA) and surface immunogenic protein (rSip) of *S. agalactiae* was shown to increase the serum IgG1 antibody titre in experimental mice immunised by an intra-mammary route (Xu et al., 2011). Not much effort in developing a vaccine against bovine mastitis due to *S. dysagalactiae* has been made either. However, the surface proteins GapC and Mig of *S. dysagalactiae* were reported to be potential protective antigens against bovine mastitis (Bolton et al., 2004).

1.9 History of vaccine development against bovine mastitis caused by *S. aureus*

The high antibiotic cost, antibiotic residues in milk and severe consequences of infection and drug resistance against *S. aureus* highlight the importance of prevention. Availability of an effective vaccine is one of the alternative methods to prevent mastitis. Many investigators have tried to develop an effective vaccine against mastitis, but none have yet yielded satisfactory outcomes (Lee et al., 1988; Greenberg et al., 1989; Mamo et al., 1994; McKenney et al., 1999; Leitner et al., 2000; Tenhagen, 2001; Leitner et al., 2003). Several approaches for vaccine development that have been undertaken have claimed to reduce the clinical severity but not prevent intra-mammary infection (IMI), of are as follows:

1.9.1 Use of whole organism as vaccine

These vaccines comprise killed bacteria or lysates emulsified either in alum-based or oil-based adjuvant. Watson for the first time in 1981 has suggested that *S. aureus* produces additional antigen *in vivo* which can be used to develop a vaccine against bovine mastitis. A heat killed *S. aureus* vaccine composed of capsular

polysaccharide of *S. aureus* and CP of *S. epidermidis* was studied for its protective potential against bovine mastitis in two herds in USA (Yoshida et al., 1984). There was remarkable decrease in leukocyte count of milk and increase in resistance to mastitis infection post vaccination for 4 months. Besides, the loss of milk production was also reduced in the vaccinated group. One of the commercially available whole organism vaccines since the 70's is lysigin that contains whole cell lysates of five *S. aureus* strains of CPS serotypes 5, 8 and 336 (Middleton, 2008) which reduces the clinical severity of mastitis. Formalin-killed autogenous vaccines have also been used over the last few decades although the results have tended to vary depending upon the virulence attributes of the endemic *S. aureus* strains involved (cited from Middleton, 2008). A field trial using a killed *S. aureus* vaccine with toxoid and composite immunological adjuvant was performed to prevent bovine mastitis in Australia (Watson and Schwartzkoff, 1990). Intramuscular administration of the vaccine in cows 8 weeks prior to calving reduced overall incidence of mastitis to 45-52% including 25% of subclinical mastitis caused by *S. aureus*. Tollersrud et al (2001) compared the immunogenicity of *S. aureus* type 5 CPS-conjugate vaccine with the killed whole cell vaccine and reported better performance for the whole cell vaccine in respect to humoral immune response. Leitner et al. (2003a) developed a vaccine, MASTIVAC 1, which after demonstrating its protective potential in non-mastitic mouse model (2003a) was evaluated its protective potential in dairy cattle in a challenge (Leitner et al., 2003b) and field trial (Leitner et al., 2003c). While the protection against one of the parent strains in the challenge trials was reported to provide significant protection, difference between the immunity efficacy of vaccinates versus the controls in the field trial was statistically insignificant. Furthermore, no analysis of the capsular types of the selected vaccine strains was carried out. Athar (2007) studied four different formalin inactivated *S. aureus* vaccines against mastitis in lactating buffaloes. These vaccines consisted of formalin inactivated *S. aureus* vaccine adjuvanted with dextran sulphate, aluminium hydroxide or both dextran sulphate plus aluminium hydroxide. Intramuscular administration of the vaccines reduced the incidence of subclinical mastitis caused by *S. aureus* accompanied by significant increase of antibody in serum and whey, increase milk yield and decreased somatic cell count.

1.9.2 Live attenuated vaccines

Another approach that has yielded interesting results is the generation of live attenuated (*aroA*) *S. aureus* that has been reported to induce significant protection in mice (Buzzola et al., 2006). Live vaccine triggered specific antibody and enhanced phagocytic activity of neutrophils by stimulating IgG_1 and IgG_2 in a study using both live and killed *S. aureus* (Davidson, 1987). A study including four *S. aureus* vaccines consisting live attenuated, dextran sulphate adjuvanted, oil adjuvanted and simple bacterin demonstrated significant reduction in somatic cell count (Shakoor et al., 2006). These vaccines included live attenuated, plain bacterins of *S. aureus* and two killed *S. aureus* vaccines with different adjuvants including dextran sulphate and mineral oil. Partial protection against mouse mastitis was reported by Buzzola et al. (2006) against challenge with the homologous parent strain although no information on the mutant *S. aureus* phenotype or resistance against challenge with various capsular types was reported.

1.9.3 Capsular polysaccharide vaccines

Yoshida et al. (1984) demonstrated that an *S. aureus* mastitis vaccine composed of capsular polysaccharide of *S. aureus* in combination with capsular polysaccharide from *S. epidermidis* increased resistance to infection upto 4 months of vaccination. Another study with *S. aureus* vaccine comprising pseudocapsule, alpha and beta toxoids of *S. aureus* with mineral oil adjuvant prevented clinical mastitis caused by *S. aureus* in the first lactation of the heifers (Nordhaug et al., 1994a). Guidry et al., 1997 suggested that regional variation in prevalence of *S. aureus* capsular polysaccharides should be considered while formulating capsular polysaccharide based mastitis vaccines. *S. aureus* isolated from human infections have been shown to produce 12 polysaccharide serotypes (11 different capsular serotypes) and one surface polysaccharide 336 (Guidry et al., 1998). However, only polysaccharides of serotypes 1, 2, 5 and 8 have been purified and characterized (O'Riordan and Lee, 2004; Jones, 2005). The majority of clinical isolates were reported to express a capsular polysaccharide of either serotype 5 or 8, representing 70-80% of the isolates from all sources (Arbeit et al., 1984; Hochkeppel et al., 1987; Poutrel et al., 1988), the rest being non-typeable. Guidry et al., 1998 reported that non-typeable bovine isolates belonged to serotype 336. The 336 antigen was characterized as polyribitol-

phosphate-*N*-acetylglucosamine, a component of cell wall teichoic acid (Verdier et al., 2007) but no data on its vaccine potential is available at present. A trivalent *S. aureus* mastitis vaccine containing formalin inactivated *S. aureus* strains belonging to serotypes 5, 8 and 336 with and without adjuvants was evaluated for its immune response in dairy cattles (Lee et al., 2005). These vaccine formulations stimulated antigen-specific IgG1 and IgG2 production in serum. Vaccine formulations with adjuvant (either alum or Freund's incomplete) stimulated even more IgG2 production. A trademarked vaccine, StaphVAX (Nabi Biopharmaceuticals, Inc) was prepared by conjugating purified CP5 and CP8 antigens to mutant nontoxic recombinant *P. aeruginosa* exotoxin A (rEPA).The Phase III trials of this vaccine to prevent *S. aureus* infections in humans have been stalled due to unfavourable outcomes (Middleton, 2008).

1.9.4 Surface and cell wall associated virulent factors as vaccines

This approach has involved use of surface proteins particularly clumping factor (Clf) A, fibronectin-binding protein (FnBP) A, protein A and a collagen-binding protein (CNA) as vaccine candidates. Mamo et al., (1994) reported that mice immunized with FnBPA were partially protected against intra-mammary challenge. However, combination of FnBP fusion protein with staphylococcal α-toxoid did not increase the efficacy of the vaccination either. Similarly, vaccination of mice with the CNA-FnBP proteins reduced the post-challenge mortality (Zhou et al., 2006) whereas dairy heifers vaccinated with these proteins alone or in combination were not effective in countering challenge with the parent strain. However, when the fusion protein was incorporated in immunostimulating complexes (ISCOMs), no clinical cases of mastitis post-intramammary challenge with 1,000 CFU of the parent strain were recorded (Nelson et al., 2006; cited from Middleton, 2008), thus raising the important question on the selection of an appropriate adjuvant for immunization of dairy cattle for effective protection against mastitis.

1.9.5 Genetic immunization

It is an attractive advancement in vaccine development that has been used to elicit potentially protective antibodies and cell-mediated immune responses in a variety of animal models of viral and bacterial diseases (Donnelly et al., 1999; Tuteja, 1999).

One of the claimed advantages of this method is that the antigen is produced *in vivo* and hence may induce both arms of the immune response (Condon et al., 1996). One recent report on the immunogenicity of a naked DNA encoding penicillin-binding protein, PBP2a, revealed the generation of a non-dose dependent antibody response to MRSA (Roth et al., 2006) in mice. A DNA vaccine encoding the clumping factor A was recently reported to yield high antibody titres in dairy cattle (Nour El-Din et al., 2006) but no challenge data was presented. However, Castagliuolo et al. (2006) reported success in providing significant protection against mastitis in the mouse model following intranasal immunization with four, rather than one, adhesins including fibrinogen-binding protein, fibronectin binding protein, clumping factor A and collagen-binding adhesion as target in a DNA vaccine. Intranasal immunization with a pDNA mixture coding the four adhesins, triggered significant levels of specific serum and mucosal Ig that inhibited *S. aureus* adhesion to cow mammary gland epithelial cells in vitro.

1.9.6 Toxoids as vaccine

There have been various efforts to develop vaccine using toxoids to prevent *S. aureus* mastitis. Kume and Murase (1976) studied the protective potential of *S. aureus* vaccine composed of bacterial toxin and reported to be effective in preventing clinical mastitis in cows. An animal trial in cows and buffaloes using alum-cell-toxoid and aluminium hydroxide gel-cell toxoid of *S. aureus* resulted prevention of subclinical mastitis in vaccinated animals (Pal and Pathak, 1977). Formulation of vaccine against *S. aureus* mastitis by using *S. aureus* toxoids with adjuvant was first suggested by Watson, 1984. A more recent approach has reported prevention of subclinical mastitis by immunizing dairy cattle by immunization with an inactivated recombinant staphylococcal enterotoxin (a superantigen) type C (SEC) single mutant vaccine administered by the intramuscular route (Chang et al., 2008). An SEC-specific ELISA test conducted at 4 wk post-immunization confirmed the presence of a high antibody titre against SEC in all vaccinated cattle. The somatic cell counts from the vaccinated group remained relatively low, whereas those of control group increased significantly after challenge with *S. aureus*. After challenge, *S. aureus* was not isolated from any cattle in the vaccinated group, whereas it was isolated from 75% of the cattle in the control group. Another study has reported success in

achieving protection against intravenous challenge with *S. aureus* in mice immunized with a double mutant of staphylococcal enterotoxin C (dmSEC) devoid of superantigenic activity in combination with cholera toxin as adjuvant by the intranasal route (Hu et al., 2006).

1.9.7 Non-capsular surface polysaccharide of *S. aureus* as vaccine candidate

The use of a surface polysaccharide, poly- N-acetyl glucosamine (PNAG), a putative biofilm-forming antigen, and demonstrated to provide at least partial protection against staphylococcal infections in mice (Maitran et al., 2004). A vaccination study using bacterins and PNAG to protect *S. aureus* mastitis in sheep demonstrated antibody production against PNAG and prevented mastitis against *S. aureus* and suggested use of PNAG as important candidate of vaccine against bovine mastitis (Perez et al., 2009).

1.10 Prospect of vaccine development against *S.aureus* considering the mode of growth

Amongst the two modes of growth by *S. aureus*, the biofilm versus planktonic, the former is difficult to treat and control as it is 50-500 times more resistant to antibiotics (Stewart and Costerton, 2001). Innate immune system of host can assist in the elimination of the planktonic form of *S. aureus* which is considered a transient state. In an *in vitro* experiment it was demonstrated that human leukocyte (lymphocytes) can actively phagocytose planktonic form of *S. aureus*, but was unsuccessful to do the same to biofilm form of *S. aureus* (Leid et al., 2002). Planktonic *S. aureus* usually stimulates antibody production in host. But due to formation of deeper layers antibodies are ineffective against biofilm. Further it leaves the immune complex of adjacent tissues damaged enough to produce antibodies (Cochrane et al., 1998). The infections caused by biofilm form of *S. aureus* are not countered by the defense mechanisms even in healthy individuals owing to this reason.

Most of the vaccine development programmes have focused on infections induced by planktonic *S. aureus* (Stranger-Jones et al., 2006; Kim et al., 2010). These vaccines

have reduced the clinical severity of *S. aureus* infections, but failed to prevent new infections (Middleton, 2008). Most of these vaccines have targeted one or a few virulence factors related to *S. aureus* infections. *S. aureus* produces almost 70 virulence factors and biofilm being one of the potent virulence factors (Costerton et al., 1999; Harro et al., 2010). Targeting to neutralize the effect of one or few virulence factors is unlikely to assist in the control of *S. aureus* infections. Besides, *S. aureus* once enters into a host is either removed by host immune mechanism or form biofilm after attaching to extracellular matrix of host. In the biofilm form, proteome of *S. aureus* is transformed into biofilm phenotype which is different from the planktonic phenotype (Brady et al., 2007). Therefore a host vaccinated against planktonic *S. aureus* antigens may eradicate only a part of biofilm and hence the inefficiency associated with the planktonic vaccines. Brady et al. (2011) developed a vaccine which is composed of four immunogenic cell-wall associated antigens which were upregulated during biofilm formation of *S. aureus* in rabbit's skin infections. The vaccine reduced radiographic scores of infection, but, failed to reduce clinical signs and clear the infection. A new approach of vaccine development against *S. aureus* should consider antigens which are common in both planktonic and biofilm mode of growth of *S. aureus* (Harro et al., 2010). It is important to choose the right components of biofilm while formulating an anti-biofilm *S. aureus* vaccine. Biofilm is mainly composed of two components: Biofilm matrix and *S. aureus* cells within biofilm. Most of the anti-biofilm vaccines have used biofilm matrix as candidates for vaccine formulation (Schaffer and Lee, 2008), PNAG being the commonest one. PNAG has shown promising results against *S. aureus* mastitis in animal models (Perez et al., 2009) and *S. aureus* abscess formation (Gening et al., 2010). However, more studies need to be conducted to establish efficacy of PNAG against *S. aureus* biofilm infections. Since the extracellular matrix of the biofilm assists the pathogen to evade immune defense mechanisms of the host, in many conditions it is produced and sloughed off (Harro et al., 2010). Thus it can be concluded that a potent anti-biofilm *S. aureus* vaccine should be composed of both the extracellular matrix and the *S. aureus* cells within biofilm which actually produces the matrix.

1.11 Objective of this research project

The objective of this research were to test the hypothesis that mice vaccinated with bacterins from strong biofilm producing *S. aureus* will generate better immune response and confer protection against mastitis following challenge with a biofilm producing *S. aureus* than a vaccine which is composed of only bacterins from planktonic form of *S. aureus.* The ultimate goal of the project is to develop a rational effective vaccine formulation for the prevention of bovine mastitis in Australia. The aims under pinning the achievement of this goal are based on the potential mechanism/processes of pathogenesis followed by *S. aureus* to establish an infection in the mammary gland (udder) of ruminant species and produce mastitis. It is contended that the first step in the production of mastitis by *S. aureus* contributing to biofilm formation is colonisation via adhesins, accompanied by immune evasion via surface-associated protein and/or polysaccharide antigens. The second step is the damage caused to the mammary tissue by via exotoxins and/or production of proinflammatory cytokines. It is therefore essential to have knowledge on the potential virulence factors detected or produced by the Australian bovine mastitis isolates. Hence the aims of this investigation were as follows:

1. Determination of biofilm forming potential and antibiograms of *S. aureus* isolated from cases of bovine mastitis in Australia.
2. Distribution of virulence factors in *S. aureus* isolated from clinical and subclinical cases of bovine mastitis in Australia.
3. Production of capsule-specific antisera for use as reagents for typing of *S. aureus* of Australian origin and determination of the prevalence of capsular polysaccharide (CPS) types of *S. aureus* isolated from clinical and subclinical cases of bovine mastitis in Australia.
4. Standardise the mouse mastitis model system for use in the assessment of the immunogenic potential of different vaccine candidates developed in this project.
5. Evaluation of immunogenicity and protective potential of *S. aureus* planktonic and biofilm- based vaccines using the mouse mastitis model system.

6. Evaluation of the immunogenicity and protective potential of the 2nd major immune evasion antigen, Protein A using the mouse mastitis model system.

Chapter 2: Methods

Every chapter in the thesis (chapters 3 to 9) contains "Materials and Methods" section as applicable to the research question embodied in different chapters. This chapter only includes those general methods which have been used during investigations described in this thesis but have not been presented elsewhere in the thesis.

2.1 Bacterial strains and culture conditions

One hundred and fifty four (154) fully characterised *S. aureus* strains of Australian origin isolated from clinical cases of mastitis in cows in Victoria and Queensland were generously donated by Professor Margaret Deighton, (RMIT University), Dr. Sharon de Wet (Queensland Biosecurity laboratory) and Dr. Justine Gibson (University of Queensland). Reference strains representing CP types 1 (strain M), 2 (strain Smith diffuse), 5 (strain Newman), 8 (USA 400 MW2) and a non-capsulated strain (LAC, USA 300) were donated by Professor Gerald Pier (Harvard Medical School, Boston, USA). *S. aureus* ATCC® 29213™, ATCC® 49775™, ATCC® 8096™ and ATCC® 55804™ were purchased from ATCC, USA. Hu *S. aureus* 11 strain from human origin was characterised and established in Associate Professor T.K. Mukkur's laboratory.

All the bacterial strains were grown on Mueller Hinton (MH) agar and subcultured in nutrient broth supplemented with 1% glucose and stored on cryobeads (Blackaby Diagnostics) or as glycerol (15%) broth stocks at -80°C. For preservation of the strains seven colonies were emulsified in cryo-preservative fluid and mixed thoroughly by inverting the vial and finally removing the cryo-preservative fluid. This was followed by storage at -80°C.

2.2 Antimicrobial susceptibility test (CDS method)

Sensitest plates (PathWest Laboratory Medicine, Western Australia) were used to perform antimicrobial susceptibility test. Overnight culture of *S. aureus* grown on MH agar plate was used for the study. Using a straight wire single colony of *S. aureus* was stabbed and inoculated in 0.9% saline. Using a Pasteur pipette saline was mixed up and down for 10 times. Sensitest plates were flooded with saline

suspension followed by rocking the plates for evenly distribution. Excess suspension was removed by using a Pasteur pipette and lid of the plates were removed and agar was allowed to dry for 10-15 min in room temperature. Plates were loaded with antibiotic disc limited to 6 discs in one plate. Plates were incubated at 35ºC for 18 h. The measurement for zone of inhibition was taken from the edge of the disc to the start zone of bacterial growth. Zone of inhibition 6 mm or greater in diameter were interpreted as sensitive except for vancomycin (VA5) and teicoplanin (TEC 15), for which, a 2 mm diameter was considered as the cut-off.

2.3 Extraction of genomic DNA

Extraction of genomic DNA was performed by using a kit (MO BIO Laboratories, Inc Carlsbad, CA). *S. aureus* strain was grown in brain heart infusion broth at 37ºC overnight. Briefly 1.8 ml of overnight grown culture was centrifuged at 10,000 x g for 30 sec at room temperature and pellet was retained. Pellet was suspended in 300 µl of microbead solution, vortexed gently and transferred to micro bead tube. Fifty microliter of MD 1 solution was added, vortexed for 10 min and centrifuged at 10,000 x g for 30 sec at room temperature. Supernatant was collected and 300 µl of MD 2 solution was added, vortexed for 5 sec and incubated at 4 ºC for 5 min. This was followed by centrifugation at 10,000 x g for 1 min. The supernatant was transferred to a new tube and 900 µl of MD 3 solution was added. The suspension was transferred to spin filter and centrifuged at 10,000 x g for 30 sec and supernatant was discarded. The pellet was suspended in 300 µl of MD 4 solution and centrifuged at 10,000 x g for 30 sec. The flow through was discarded and the spin filter was placed in a new 1.5 ml collection tube and 50 µl of MD 5 solution was added to the centre of the filter which was followed by centrifugation at 10,000 x g for 30 sec. The spin filter was discarded and DNA was stores at -20°C until use.

2.4 Detection of *mec*A and *pvl* gene by using Genotype-MRSA kit

Detection of *mec*A and *pvl* gene was performed by using Genotype-MRSA kit (Hain Lifescience). The test is based on DNA strip technology. Briefly, 45 µl of amplification mix was prepared by mixing 35 µl PNM, 5 µl 10x polymerase incubation buffer, 3 µl MgCl2 solution, 1.6 µl nuclease free water and 0.4 µl DNA polymerase. To the mixture 5 µl of genomic DNA was added to make a total volume

of 50 µl. The amplification conditions were 95°C for 5 min, 22 cycles each of 95°C for 20 sec and 60°C for 30 sec. The amplified product was stored at -20°C until the next step. The final step was hybridization biotin-labelled amplicons to membrane-bound probes. This was achieved by Dispensing 20 µl of denaturation Solution to each well of the tray provided with the kit and adding 5-20 µl of amplified product. This was followed by incubation for 5 minutes at room temperature. One ml of hybridization buffer was added to each well and mix properly with gentle shaking. To each well a strip (provided with the kit) was placed and incubated at 45°C in shaking water bath for 30 min. After incubation hybridization buffer was aspirated and 1 ml of stringent wash solution was added to each strip and was further incubated at 45°C in shaking water bath for 15 min. Wash solution was poured out and each strip was washed with 1 ml of Rinse Solution for 1 minute and 1ml of conjugate was added and incubated for 30 min at the same temperature and condition. The strips were washed twice for 1min using 1ml each of rinse solution and distilled water. Finally water was poured out from the wells and 1 ml of substrate was added to each well and incubated the wells at 45°C for 20 min. The reaction was stopped by using distilled water and dried the strips by using absorbent paper. Evaluation and interpretation of results is done as per the bands developed in the strip. The strip has 6 reaction zones which are shown below in a diagram.

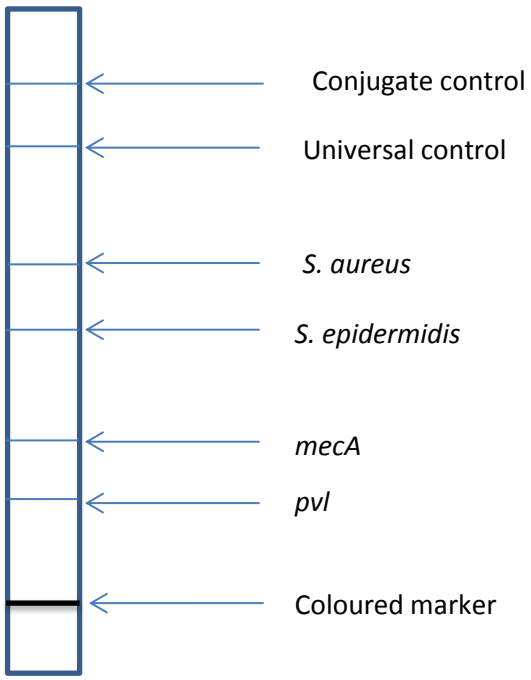


Figure 2.1: Reaction zones in the strip of Genotype MRSA kit

2.5 Preparation of splenocytes for estimation of IFN-γ levels post-stimulation with experimental or control antigens

Mice were euthanized by using cervical cordotomy in anaesthetised state and spleens were collected in ice-cold 2 ml of Dulbecco's modified Eagle's medium (CDMEM, Gibco) without adding fetal bovine serum (FBS). Spleens were ground on a 70µm cell strainer (BD) using a 10ml syringe piston and cells were collected in a 50ml falcon tube with a final rinse of 3ml of CDMEM without FBS. Splenocytes were pelleted at 1000 x g (Beckman Coulter Avanti® J-E) for 10 min which was followed by re-suspension of pellet in 3 ml of CDMEM without FBS and 500 µl of ammonium chloride was added to the splenocyte suspension for lysis of RBC. The suspension was incubated at room temperature for 2 min and centrifuged at 1000 x g for 10 min. Cells were washed twice using 3 ml of CDMEM without FBS at 1000 x g for 10 min. The pellet was resuspended in 10 ml of complete DMEM media with 10% FBS, 100 µg/ml streptomycin/ml and 100 U penicillin/ml and centrifuged at 1000 x g for 10 min. The viability of splenocytes were checked by aliquoting out 10 µl of suspension and counting using a haemocytometer where staining of the cells was done with trypan blue. Haemocytometer (Neubauer) with a depth of 0.1 mm with conversion factor 10,000 was used to perform viable cell count. Ten ml of splenocytes was diluted 1: 10 with CDMEM. Trypan blue (Invitrogen) in equal proportion was added to get a dilution factor of 1:20. The chambers of haemocytometer were filled with the suspension by capillary action and average counting was performed for all the four large outer squares. The average number of cells/ml was multiplied by dilution as well as conversion factors to get the total number of viable cells per ml of splenocytes. The concentration of cell was adjusted to $1x10^7$ splenocytes/ml. One ml of CDMEM with 10% FBS was taken in each well of 24 well tissue culture plate and 1ml of splenocyte suspension was added to get a final cell concentration of 5×10^6 splenocytes/well.

Selected *S. aureus* antigens (killed biofilm *S. aureus*, killed planktonic *S. aureus*, ProteinA) were added in duplicate wells and cells were incubated at 37ºC with 5% CO_2 for 72 h. The wells were checked every 24 h for cloudiness which indicates contamination and contaminated wells if any were discarded. After completion of 72

h supernatant from each well was collected for measurement of IFN-γ production. The splenocyte supernatants were stored at -80ºC for further study.

2.6 Preparation of antigens for stimulation of splenocytes

For both planktonic and biofilm vaccines, single colony of overnight grown BOAISRF *S. aureus* 51 strain was inoculated into each well of 24 well flat bottoms with lid tissue culture plates (Sarstedt Australia Pty Ltd, SA) which were filled with 1.5 ml of 1% glucose enriched nutrient broth. Plates were incubated at 37º C for 24 h with gentle shaking at 80 rpm. Planktonic form of antigen was obtained by collecting the supernatant using a pipette and centrifuged at 10,000 x g for 10 min. The biofilm was collected by scooping out with sterile spatula and washed using 1ml of sterile PBS. Both the planktonic and biofilm forming cells were washed for five times at 10,000 x g for 10 min by using PBS. Cells were suspended in 5 ml of PBS to which 2 ml of 3% formalinized PBS was added and allowed to remain at room temperature for a period of 24 h. This was followed by centrifugation at 10,000 x *g* for 20 min and washing with PBS five times and resuspending in 5ml of sterile PBS. Based on the growth curve the desired colony forming units of BOAISRF *S. aureus* 51 in both planktonic and biofilm form were adjusted to 1 x 10^8 cfu/ml. For ProteinA vaccine, 2.5 µg of purified protein A was prepared for addition to each well of splenocyte suspension.

Chapter 3: Analysis of biofilm forming potential, antibiotic resistance and related genes of Australian bovine mastitis-associated *Staphylococcus aureus* isolates

Prologue

One of the prerequisites for establishment of infection with most pathogens is colonisation of the target site, followed by formation of biofilms. Biofilm formation in the mammary gland of cows infected with *S. aureus* should be no exception. Biofilm formation by pathogens has been reported to bestow the property of enhanced resistance to antibiotics and phagocytosis (Matz et al., 2005; Bjarnsholt, 2013). Biofilm formation creates a major obstacle in the treatment and prevention of bovine mastitis caused by *S. aureus*.

3.1 Introduction

Biofilm is the aggregation of bacterial colonies attached to biotic or abiotic surface and being enclosed by self-produced extracellular matrix. It is mainly composed of two components- micro colonies of *S. aureus* cells (≥15% by volume) and extracellular matrix (≥85%) [Kokare et al., 2009]. Formation of biofilm by *S. aureus* helps the bacterium to acquire nutrition (Decho, 1990) and new genetic traits (Brady et al., 2007). Besides, it protects the pathogen from host immune defence mechanism and antimicrobial agents (Monzon et al., 2002). Acute mastitis is usually caused by planktonic *S. aureus* which is easy to treat with antibiotics depending on the accuracy and promptness of diagnosis (Bjarnsholt, 2013). However, *S. aureus* succeedes in forming a biofilm does not respond to antibiotic treatment and develops into chronic form of mastitis (Bjarnsholt, 2013).

Formation of *S. aureus* biofilm can be described in three steps: attachment, maturation and detachment (Otto, 2013). Attachment of *S. aureus* cells to mammary tissue occurs due to interaction of surface anchored bacterial factors, particularly the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) including fibronectin binding proteins (FnBPA, FnBPB), fibrinogen binding proteins (ClfA, ClfB), collagen binding protein (CNA) and bone sialo binding protein (BBP) (Patti et al., 1992; Mc Devitt et al., 1994; Tung et al., 2000 and O'Neill et al., 2008). The second step of biofilm development is the formation of polysaccharide

intercellular adhesion (PIA), alternatively referred to as PNAG (Poly-β-1, 6-linked *N*-acetylglucosamine) which is activated by four proteins including IcaA, IcaD, IcaB and IcaC produced by single *icaADBC* operon, IcaA and IcaD being the most important proteins for the production of PNAG (Otto, 2009). The role played by PNAG to form biofilm in *S. aureus* infections has been demonstrated (Maira- Litran et al., 2004). However, few recent studies have demonstrated the production of biofilm by even PNAG- negative strains with the help of other adhesion proteins of *S. aureus* including proteinA (Merino et al., 2009) and biofilm-associated protein [bap] (Lasa and Penades, 2006). The third step in biofilm formation is the detachment of biofilm which facilitates dissemination of *S. aureus* from the original site of infection to other tissues or surfaces (Gil et al., 2014). An accessory gene regulator (*agr*) can negatively influence biofilm production contributing to the detachment of biofilm and dispersion (Boles and Horswill 2011). Among the four agr types (Goerke et al., 2003), specific types appear to be associated with certain phenotypes, including antibiotic resistance and biofilm production (Manago et al., 2006). It has been demonstrated that specific agr type is associated with particular infections caused by *S. aureus.* Agr type 1-IV is associated with enterotoxigenic infections and endocarditis, endocarditis, toxic shock syndrome toxin and exfoliative disease, respectively (Jarraud et al., 2002).

Different phenotypic and genotypic methods have been used to determine the ability to produce biofilm by *S. aureus*. The common phenotypic methods used are Congo red agar (CRA) method, Tissue culture plate (TCP) method, Tube method (TM) and the Transmission and Scanning Electron microscope method. The genotypic method includes the detection of intercellular adhesion genes (*icaA, icaD, icaB* and *icaC*), *bap* gene by using conventional polymerase chain reaction (PCR). Different investigators have reported different results regarding the effectiveness of biofilm production detection methods. Dhanawade et al (2010) reported the CRA method to be superior to the TCP and TM methods as TCP method identified 48.03% of biofilm producers, followed by 36.27% by TM method and 19.60-24.91% by TCP method. Similarly Vasudevan et al (2003) confirmed the superiority of the CRA methods over the other methods and reported the identification of 91.4% *S. aureus* strains as biofilm producers using the CRA method. In contrast, the TCP method was found to be superior detecting 57.8% of the strains as biofilm producer (Mathur et al., 2006).

These authors could not differentiate negative biofilm or weak biofilm producers using the TM method. Besides the CRA method did not show any correlation between TCP and TM methods in regards to biofilm formation.

The antimicrobial tolerance of *S. aureus* biofilms may be due to factors like (a) presence of extracellular matrix (Kolpen et al., 2010) (b) decreased growth rate leading to smaller size of cells which are less susceptible to antibiotics (Thein and O'Toole, 2001) (c) presence of antibiotic degrading enzymes in biofilms causing inactivation of antibiotic molecules trying to enter into biofilm (Raza et al., 2013) and (d) change in the protein structure of cell wall of *S. aureus* in biofilms than the planktonic mode of growth (Potera, 1999). Ability to evade host defence mechanism and resist antibiotics and antimicrobial agents make *S. aureus* biofilm-associated infections untreatable and transforms the infection to chronic state (Cucarella et al., 2004). There are no studies to the best of our knowledge; on either the development of persistence of biofilm-associated antibiotic resistance of *S. aureus* or the emergence of resistance to different antibiotics. The major aim of this study is to investigate: (a) the antimicrobial susceptibility pattern of *S. aureus* isolates from clinical cases of bovine mastitis (b) the biofilm forming potential of these isolates and the impact on emergence of persistent antibiotic resistance developed by selected clinical isolates of *S. aureus* or (c) inter-relationship between production of biofilm by these isolates and presence of *ica*, *bap*, *blaZ* or *agr* genes in these isolates.

3.2 Material and Methods

3.2.1 *S. aureus* isolates

One hundred and fifty four (154) fully characterised *S. aureus* strains of Australian origin isolated from clinical cases of mastitis in cows in Victoria and Queensland were used for the study. Reference strains representing CP types 1 (strain M), 2 (strain Smith diffuse), 5 (strain Newman and USA 100 NRS 648), 8 (USA 400 MW2) and non-capsulated strains (LAC, USA 300 and USA 300 NRS 648) and a strong biofilm former ATCC 29213 were used as positive controls. BOAISRF *S. aureus* 15, 25, 114, 131 1nd 146 were characterised and established in our laboratory were used as positive control for *bap* and *agr* types (Table 3.1). These isolates were grown on Mueller Hinton (MH) agar and subcultured in nutrient broth supplemented

with 1% glucose and stored on cryobeads (Blackaby Diagnostics) or as glycerol (15%) broth stocks at -80°C.

3.2.2 Screening of *S. aureus* isolates for antibiotic resistance

One hundred and fifty four (154) strains of *S. aureus* were screened for their antibiogram by using CDS method (Bell et al., 2009). The antibiotics used in the experiment were benzylpenicillin (P 0.5), 0.5µg, cefoxitin (FOX 10), 10µg, cephalexin (CL 100), 100µg, ciprofloxacin (CIP 2.5), 2.5µg, co-trimoxazole (SXT 25), 25µg, erythromycin (E 5), 5µg, linezolid (LZD 10), 10µg, mupirocin (MUP 200), 200ug, rifampicin (RD 1), 1µg, teicoplanin (TEC 15), 15µg, tetracycline (TE 10), 10µg and vancomycin (VA 5), 5µg (Oxoid). Sensitest plates (PathWest) were used to subculture *S. aureus* and plates were incubated at 37°C for 18 h after which the antibiotic sensitivity or resistance was recorded as described elsewhere, with the measurement taken from the edge of the disc to the start zone of bacterial growth (Bell et al., 2009). Zones of inhibition of 6 mm or greater in diameter were interpreted as sensitive. Exceptions to this rule were for vancomycin (VA5) and teicoplanin (TEC 15), for which, a 2 mm diameter was used as the cut-off.

To determine the persistence or emergence of antibiotic resistance, 28 biofilm forming (weak, intermediate and strong) *S. aureus* strains were selected and following the same protocol antibiotic resistance study was performed for both planktonic and biofilm forms of these *S. aureus* against the above mentioned 12 antibiotic agents. These strains were all tested as sensitive against all the 12 antibiotics in planktonic form.

3.2.2.1 Screening of *S. aureus* isolates for methicillin resistance

Detection of methicillin resistance of the 154 strains was detected by 3 different methods: Chromogenic MRSA ID plates, Use of MRSA kit and PCR analysis of *mecA* gene.

3.2.2.1.1 Use of chromogenic culture media plates

All isolates were streaked on Chromogenic MRSA ID plates to check for bacterial ability to grow in the presence of cefoxitin (Biomerieux, 2011). Chrom ID MRSA

plates [Biomerieux] use cefoxitin to assess resistance to methicillin, in combination with an antibacterial compound, which inhibits growth of MSSA and other bacteria. Colonies showing green blue pigment are classified as MRSA positive whereas MSSA strains do not grow on these plates.

3.2.2.1.2 Detection of *mec*A gene by using Genotype-MRSA kit

As per the standard protocol provided with the Genotype-MRSA kit (Hain Lifescience) detection of *mecA* gene was accomplished as described in Chapter 2 Section 2.4. The positive control used for *mecA* was Strain Newman (CP5).

3.2.2.1.3 Detection of *mec*A gene

To reconfirm the results acquired by the above two methods, identification of *mecA* gene was done using conventional PCR (Murakami et al., 1991). The positive control used for *mecA* was Strain Newman (CP5). The PCR conditions for *mecA* was 95°C for 5 min, 35 cycles of 95°C for 30 sec, Tm for 30 sec and 72°C for 45 sec with a final extension of 72°C for 10min. The PCR product was analysed by agarose gel (1.5%) electrophoresis, staining with 0.8uL/100mL of Midori Green DNA Stain (Nippon Genetics) using 1x SB Buffer. An O'RangeRuler DNA Ladder (100-1500 bp, Fermentas) was used for comparing the approximate band sizes after visualising on UV transilluminator.

3.2.3 Detection of biofilm production

3.2.3.1 Congo red agar (CRA) method

Detection of biofilm production on CRA plates was determined by a method described elsewhere (Freeman et al., 1989) with slight modification in the incubation time by increasing to 96h to ensure maximal biofilm formation. Briefly, Isolates were inoculated on CRA plates and incubated for 96 h at 37^{o}C. Black, rough crystalline colonies were considered positive for biofilm production, and red/pink, rough crystalline or smooth colonies were considered as non-biofilm former.

3.2.3.2 Tissue culture plate (TCP) method

Aliquots (200uL) of nutrient broth, supplemented with 1% glucose, were inoculated with *S. aureus* strains and placed in a 96-well sterile microtitre plate (Kogan et al., 2005). The plates were left overnight in a 37°C incubator to ensure adequate biofilm

production. This was followed by assessment of their biofilm-forming property using the following methods:

3.2.3.2.1 Treatment with sodium metaperiodate

Sodium metaperiodate was used for treatment of the biofilms as it is known to disrupt PNAG-dependent biofilm formulation (Pozzi et al., 2012). This method was carried out as described by Kogan et al (2005). Briefly, inoculated TCP wells in the micro titre plate were washed with 200uL of 0.9% NaCl and then filled with 100uL of 10mM sodium metaperiodate in 50mM sodium acetate buffer. The microtitre plate was then incubated at 37°C for 2 hrs after which the wells were washed again with 200uL of 0.9% NaCl. The plate was dried for 45 min at 55°C and then stained for 5 mins with 5% safranin. The plate was once again washed with 0.9% NaCl and the results viewed for optical density on a micro titre plate reader at 492 nm. The assay was repeated three times and the data was averaged.

3.2.3.2.2 Crystal violet staining

This method, which measures the amount of biofilm attached to the polystyrene plate, was an adaptation of the procedures described elsewhere (Christensen et al., 1985; Mathur et al., 2006). *S. aureus* strains were grown to produce biofilm in a 96-well micro titre plate. The plates were washed with PBS (pH 7.2) to remove planktonic or free-floating bacteria (Mathur et al., 2006). The biofilm was fixed at 55°C for 10 mins and stained with 2% crystal violet. Biofilms were destained with alcohol and viewed on a microplate reader at 570 nm. The assay was repeated three times and the data was averaged. The cut-off point used for non-biofilm producing strains was 0.120 as suggested by Christensen et al (1985). An arbitrary classification system for quantification of biofilm formation (Stepanovic et al., 2000) rendered more stringent was adopted. The arbitrary cut-off points for weak, intermediate and strong biofilm formers were 0.130-4.00 x 0.120 A_{570nm}, 4.10-5.90 x 0.120 A_{570nm} and 6 x 1.20 $A_{570nm,}$ respectively.

3.2.4 Detection of *icaA, icaD, bap, agr* types, and *blaZ* genes using conventional PCR

3.2.4.1 Extraction of genomic DNA

Genomic DNA was extracted from 154 *S. aureus* isolates using a kit (MO BIO

Laboratories, Inc Carlsbad, CA) as described in Chapter 2 Section 2.3. The extracted genomic DNA was stored at -20°C until use.

Table 3.1: Amplification of biofilm related genes of *S. aureus* isolates from bovine mastitis in Australia using conventional PCR

Target gene	Positive control	Primer Forward (5'-3')	Primer Reverse (5'-3')	Tm	Expected band size (bp)	Reference
***ica*A**	USA 400 strain	CCT AAC TAA CGA AAG GTA G	AAG ATA TAG CGA TAA GTG C	48°C	1315	Vasudevan et al. 2003
***ica*D**	USA 400 strain	AAA CGT AAG AGA GGT GG	GGC AAT ATG ATC AAG ATA C	47°C	381	Vasudevan et al. 2003
bap	BOAISRF S. aureus 146	CCC TAT ATC GAA GGT GTA GAA TTG CAC	GCT GTT GAA GTT AAT ACT GTA CCT GC	42°C	971	Cucarella et al., 2001
agrI	BOAISRF S. aureus 131	ATG CAC ATG GTG CAC ATG	GTC ACA AGT ACT ATA AGC TGC GAT	60°C	441	Gilot et al., 2002
agrII	BOAISRF S. aureus 114	ATG CAC ATG GTG CAC ATG	TAT TAC TAA TTG AAA AGT GGC CAT AGC	60°C	575	Gilot et al., 2002
agrIII	BOAISRF S. aureus 131	ATG CAC ATG GTG CAC ATG	GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	60°C	323	Gilot et al., 2002
agrIV	S. aureus 15	ATG CAC ATG GTG CAC ATG	CGA TAA TGC CGT AAT ACC CG	60°C	657	Gilot et al., 2002
blaZ	BOAISRF S. aureus 25	ACT TCA ACA CCT GCT GCT TTC	TGA CCA CTT TTA TCA GCA ACC	60°C	173	Melchior et al., 2000

3.2.4.2 Detection of genes encoding *ica*A, *ica*D, *bap, agr* types, and *blaZ* genes

PCR was conducted to detect *ica*A, *ica*D, *bap, agr* type I-IV, and *blaZ* genes of *S. aureus* in 154 *S. aureus* isolates from clinical mastitis cases in cows. The primers, Tms, band size for all the genes are provided in Table 3.1 with respective references. Following are the conditions used for detection of different genes:

The PCR amplification conditions for *ica*A and *ica*D were 95°C for 5mins, 95°C for 45secs, Tm for 45secs, 72°C for 5mins with 30 cycles and final extension at 72°C for 5 mins. The cycling conditions for *bap, blaz* and *agrI-IV* types were 94°C for 5 min, 30 cycles of 94°C for 30 sec, Tm for 30 sec and 72°C for 60 sec with a final extension of 72°C for 10 min. All PCR products were analysed by agarose gel (1.5%) electrophoresis, staining with 0.8uL/100mL of Midori Green DNA Stain (Nippon Genetics) using 1x SB Buffer. An O'RangeRuler DNA Ladder (100-1500 bp,

Fermentas) was used for comparing the approximate band sizes after visualising on UV transilluminator.

3.2.5 Statistical analysis

Two-way analysis of variance (ANOVA) using Windows 2007 was performed to assess the significance of association of each of the genotypes with biofilm positive isolates.

3.3 Results

3.3.1 Screening of *S. aureus* isolates for antibiotic resistance

Antibiotic resistance study of 154 *S. aureus* strains against 12 different antimicrobial agents revealed all (100%) strains to be sensitive to cefoxitin and co-trimoxazole. Only 1 (0.65%) strain each was found resistant against cephalexin, tetracycline, vancomycin, rifampicin and linezolid, respectively. The resistant pattern against ciprofloxacin, erythromycin, Teicoplanin and mupirocin was 3.25%, 2.6%, 1.3%, 1.3%, respectively. The maximum resistance was shown against penicillin which was recorded to be 18.83%. The detail result of antibiotic resistance study against 12 different antibiotics is presented in Table 3.2 and figure 3.2.

3.3.2 Screening of *S. aureus* isolates for methicillin resistance

All the strains of *S. aureus* were found to be sensitive to methicillin in all the three tests performed viz, use of chromogenic culture media plates, Genotype-MRSA kit and PCR method.

3.3.3 Detection of biofilm production

Only 48 (31.17%) strains of *S. aureus* out of 154 were found to be positive for biofilm formation in CRA method. However, all the 154 (100%) strains were found be positive for biofilm formation which were graded as weak (74.68%), moderate (13.64%) and strong (11.69%) biofilm producer in TCP method. Detail results are presented in Table 3.3 and figure 3.1. This indicated the superiority of TCP method over CRA method for detection of biofilm formation by *S. aureus* isolates.

Table 3.2: Detection of antibiotic sensitivity of planktonic *S. aureus* (n=154) isolates from cases of bovine mastitis in Australia

Sl. No	Antibiotic (abbreviation, amount)	No. of isolates resistant	Percentage of isolates resistant
1	Cephalexin (CL 100 μg)	1	0.65%
2	Cefoxitin (FOX 100 μg)	0	0%
3	Ciprofloxacin (CIP 2.5 μg)	5	3.25%
4	Erythromycin (ES μg)	4	2.6%
5	Teicoplanin (TEC 15 μg)	2	1.3%
6	Tetracycline (TE 10 μg)	1	0.65%
7	Penicillin G (P 0.5 μg)	29	18.83%
8	Mupirocin (MUP 200 μg)	2	1.3%
9	Vancomycin (VA 5 μg)	1	0.65%
10	Rifampicin (RD 1 μg)	1	0.65%
11	Linezolid (LZD 10 μg)	1	0.65%
12	Co-trimoxazole (SXT 25 μg)	0	0%

Table 3.3: Detection of biofilm formation by *S. aureus* isolates by using CRA and TCP methods

Method of detection of biofilm	Total number of isolates tested	Total number of isolates positive	Percent of isolate positive (%)
Congo Red Agar	154	48	31.17
Tissue Culture Plate	154	SB*=18 (11.69%) IB*=21 (13.64%) WB*=115 (74.68%)	100

*SB= Strong biofilm, IB= Intermediate biofilm, WB= weak biofilm

3.3.4 Detection of *icaA, icaD, bap, agr* types, and *blaZ* genes

To determine the genetic markers of biofilm formation all the 154 strains were subjected to PCR study. The results of PCR study are compiled in Table 3.4 and 3.5. A high percentage of isolates were positive for *icaA* (70.78%) and *icaD* (83.12%). However, only 2 (1.3%) strains were positive for Bap. The prevalence *blaZ*, *agrI*, *agrII*, *agrIII* and *agrIV* in 154 *S. aureus* isolates was 20.2%, 51.3%, 1.3%, 29.9% and o%, respectively.

Table 3.4: Distribution of biofilm formation related genes in *S. aureus* isolates of bovine mastitis origin in Australia

Target gene	Total number of *S. aureus* strains positive	Percent from total samples (%)
*ica*A	109	70.78
*ica*D	128	83.12
Bap	2	1.3
blaZ	31	20.2
agrI	79	51.3
agrII	2	1.3
agrIII	46	29.9
agrIV	0	0

3.3.5 Comparison of Antibiotic susceptibility for biofilm and planktonic *S. aureus*

Antimicrobial susceptibility studies conducted on biofilms of 28 selected *S. aureus* isolates revealed multiple antibiotic resistances when grown as biofilms. All these strains were tested as sensitive to all the 12 antibiotics in planktonic form. However, once biofilm is formed 3 (10.71%) strains of *S. aureus* (BOAISRF S.aur 32, BOAISRF S.aur 40, BOAISRF S.aur 41) developed multiple antibiotic resistances including cefoxitin, erythromycin, tetracycline, vancomycin, ciprofloxacin, co-trimoxazole and teicoplanin (Table 3.6). To determine whether the developed resistance was transient or persistent, *S. aureus* cells were taken out from biofilm of these 3 strains and stored at 4ºC and re-tested for antibiotic susceptibility against the

same antibiotics on 7, 14, 21 and 30 days. The antibiotic resistance was found to be persistent upto 3 to 4 weeks and revert back to susceptibility mode after 4 weeks.

Table 3.5: Comparison (%) of the shared pairs of genotypes among *S. aureus* isolates (n=154)

Criterion	TCP	*ica*A	*ica*D	*bap*	*agr*I	*agr*II	*agr*III	*blaZ*
TCP	100							
***ica*A**	70.78	100						
***ica*D**	83.12	64.94	100					
bap	1.3	0.65	1.3	100				
agrI	51.3	33.12	48.70	0.65	100			
agrII	1.3	0.65	0.65	0	0	100		
agrIII	29.9	24.03	33.77	0	0.65	0	100	
blaZ	20.2	13.64	17.53	0	9.74	0	6.49	100

Table 3.6: Emergence/persistence of antibiotic resistance by *S. aureus* isolates in biofilm

Antibiotic	BOAISRF S.aur 32	BOAISRF S.aur 40	BOAISRF S.aur 41	BOAISRF S.aur 50
Cefoxitin (Fox 10)	**S to R***	S	S	S
Cephalexin (CL 100)	S*	S	S	S
Ciprofloxacin (CIP 2.5)	S	S	**S to R**	S
Erythromycin (E 5)	**S to R**	S	S	S
Teicoplanin (TEC 15)	S	S	**S to R**	S
Tetracycline (TE 10)	**S to R**	**S to R**	**S to R**	S
Penicillin G (P 0. 5)	S	S	S	S
Mupirocin (Mup 200)	S	S	S	S
Vancomycin (VA5)	S	**S to R**	**S to R**	S
Rifampicin (RD 1)	S	S	S	S
Linezolid (LZD 10)	S	S	S	S
Co-trimoxazole (SXT 25)	S	S	**S to R**	S

* S= Sensitive, R= Resistant

3.3.6 Statistical analysis

There was no correlation detected between biofilm formation and presence of *ica* loci, bap gene and *agr* types.

3.4 Discussion

In the present study 154 strains of *S. aureus* isolated from clinical cases of mastitis were evaluated for their ability to produce biofilm and also studied the role of five different genes such as *ica*A, *ica*D, *bap*, *agr* types and *blaZ* in formation of biofilm. Comparative study of CRA and TCP method for detection of biofilm formation by using 154 strains of *S. aureus* revealed that TCP method could determine biofilm forming ability of 100% of the strains. Whereas CRA method could only detect only 48 (31.17%) biofilm producing strains proving superiority of TCP method over CRA method. Various researchers have reported the effectiveness of TCP method ranging from 30-68.57% (Vasudevan et al., 2003; Dhanawade et al., 2010).

Most of the *S. aureus* strains used in this study were sensitive to the 12 different antibiotics used to perform antimicrobial susceptibility test. A total of 29 (18.83%) *S. aureus* strains were found to be resistant against penicillin which is followed by 3.25% and 2.6% resistance against ciprofloxacin and erythromycin. All the *S. aureus* strains were found to be methicillin sensitive. However, interesting results of antibiotic resistance pattern could be seen in the *S. aureus* cells grown in biofilm. In the present study out of 28 selected *S. aureus* strains which were initially susceptible to 12 different antibiotics after formation of biofilm and the resistance persisted as long as 4 weeks. Various investigators have reported about development of antibiotic resistance in biofilms (Monzon et al., 2002; Melchior et al., 2006; Kwon et al., 2008). However, this study has demonstrated that antibiotic resistance persist up to 4 weeks and then revert back to antibiotic susceptible state. Transformation of antibiotic sensitivity to antibiotic resistant trait is a serious concern in the area of prevention and control of Staphylococcal infections including bovine mastitis. Based on this study it can be suggested that while performing antibiotic susceptibility test for treatment of bovine mastitis both planktonic and biofilm states of growth should be tested.

There are various reports on formation of biofilm by *S. aureus* which correlates with the presence of *ica* locus (Cafiso et al., 2004; Melchior et al., 2009). In the present study, >70% isolates producing biofilm were found to carry both *ica*A and *ica*D genes. However, statistical analysis of data showed that the presence of *ica* locus was not statistically different between biofilm formation by the isolates. This finding is supported by several studies which have reported weak/no correlation between the presence of *ica* locus and biofilm formation (Yazdani et al., 2006; Dhanwade et al., 2010; Szweda et al., 2012). Only 2 (1.3%) isolates were found to be carrying bap gene which is similar with previous researchers (Cucarella et al., 2004; Nemati et al., 2009; Tang et al., 2013) who also reported rare prevalence of this gene in *S. aureus* isolated from bovine mastitis and poultry-associated infections. Presence of *agr* gene in *S. aureus* is associated with biofilm formation and antibiotic resistance, particularly against penicillin. Of the 4 *agr* types *agrI* and *agrII* are the most important types. *agrI* is associated with penicillin resistance and persistence of resistance, *agrII* is associated with antimicrobial susceptibility and biofilm formation (Manago et al., 2006; Mirani et al., 2013). *agrIII* is associated with non-invasive infections (Ben Ayed et al., 2006) and most of the exfoliatin producing *S. aureus* strain come from *agrIV* (Jarraud et al., 2000). In the present study, majority of the *S. aureus* strains produced *agr* type I (51.3%) and III (29.9%) and only 2 strains (1.3%) produced *agr* type II. None of the strains produced *agr* type IV. This finding is in accordance with Bibalan et al (2014) who has reported incidence of 43.3% and 28.87% of *agrI* and *agrIII*, respectively from *S. aureus* isolates. Resistance to penicillin is reported to be mediated by *blaZ* gene (Melchior et al., 2009). In the present investigation a total of 31 (20.2%) strains of *S. aureus* were found to carry *blaZ* gene and 29 (18.83%) strains of *S. aureus* showed resistance against penicillin. There appears to be a possible correlation between *blaZ* gene and penicillin resistance according to the data generated by this investigation. However, *blaZ* cannot be the single reason for dividing bovine mastitis-associated *S. aureus* into penicillin resistant and susceptible strains (Melchior et al., 2009). The present study could not find co-relation between biofilm formation and presence of *ica* loci, bap gene and *agr* types. However, the study emphasizes the need for further research on genetic makeup of *S. aureus* isolates related to biofilm formation and antibiotic susceptibility phenotypes. This information could assist in delineating the mechanism or regulation of antibiotic resistance which would impact the persistence

and therapeutic measures to be adopted to control the pathogen in an individual animal as well as at the herd level.

Figure 3.1A: Detection of biofilm by Tissue culture plate method

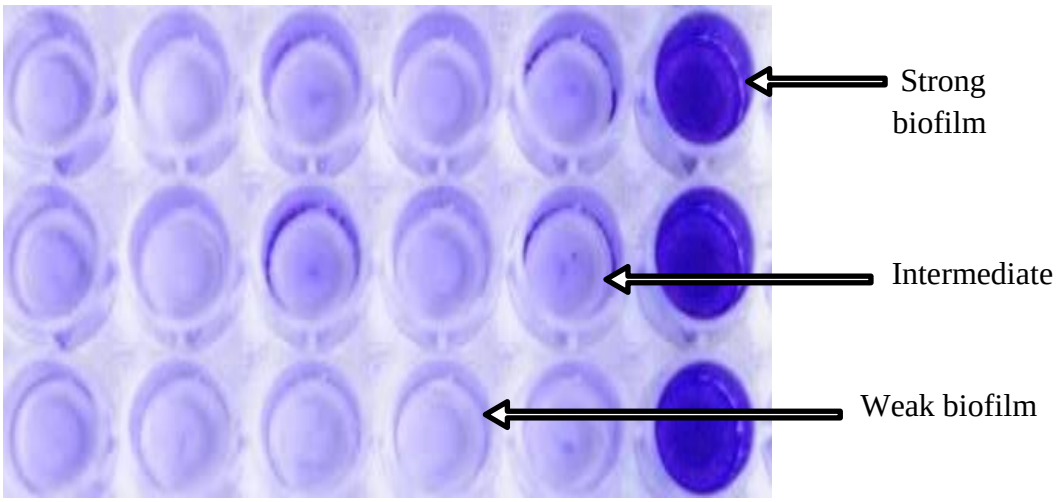


Figure 3.1B: Growth of biofilm on micro titre plate

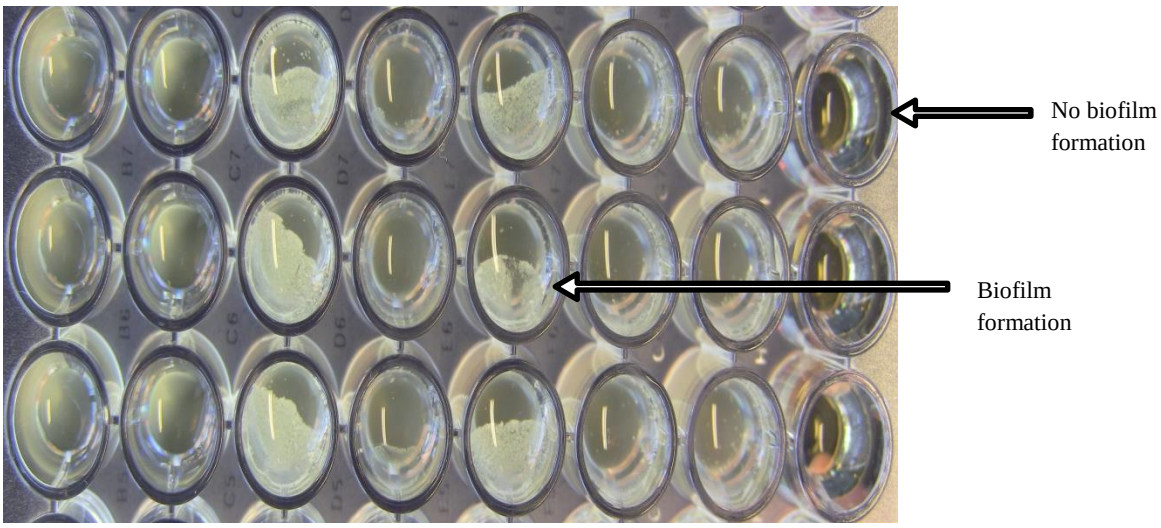
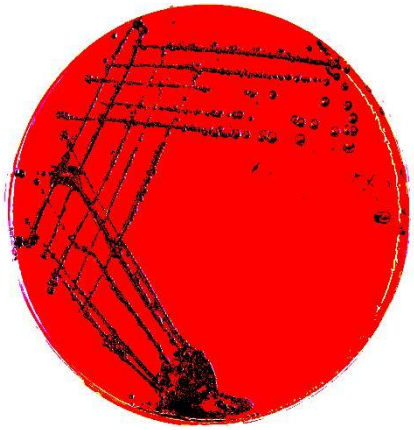


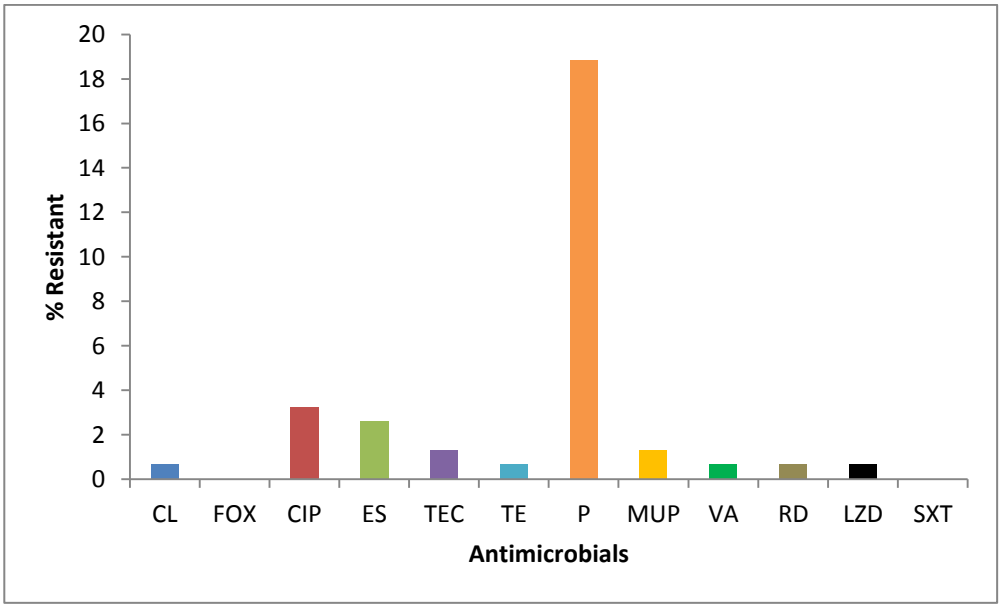
Figure 3.1 C: Detection of biofilm formation in CRA plates

Biofilm negative *S. aureus*



Biofilm positive *S. aureus*

Figure 3.2: Comparison of antimicrobial resistance against different antibiotics by planktonic *S. aureus* isolates



Chapter 4: Detection of virulence factors in *Staphylococcus aureus* isolated from clinical and subclinical cases of bovine mastitis in Australia

4.1 Introduction

Staphylococcus aureus is a well-known commensal of nares and skin of both human and animals (Wertheim et al., 2004). It can cause variety of infections when it crosses the epithelial barrier affecting almost all the organs of body. There have been numerous advances in the area of prevention and control of bovine mastitis. However, *S. aureus* is still the major contagious agent of bovine mastitis worldwide (Athar, 2007; Im et al., 2014). It causes contagious clinical or subclinical mastitis with an increase in the somatic cell count (SCC) in milk comprising macrophages, lymphocytes and neutrophils. It has been reported to cause more than 80% of subclinical mastitis cases in bovines causing a loss of US $300 per animal per year (Wilson et al., 1997; Karahan et al., 2011).

S. aureus enters the mammary gland via teat orifice through chapped or injured teats. Once the organism enters into the mammary gland, it adheres to epithelial lining and invades the host immune system by variety of antiphagocytic factors including capsule, proteinA, and fibronectin binding proteins (Patti et al., 1992; Nanra et al., 2013). Once an intra-mammary infection is established, damage to the mammary gland epithelial lining is initiated by ulceration and occlusion of lactiferous ducts and alveoli, infiltration of inflammatory cells in the parenchyma (Paape et al., 2002). Mammary tissue damage is further complicated by various toxins produced by *S. aureus* which act as super-antigens (Ikawaty et al., 2010).

S. aureus produces a variety of virulence factors by which it causes damage to host leading to signs and symptoms of disease. These virulence factors evade host immune mechanism by adhering to eukaryotic membrane, resisting phagocytosis and killing eukaryotic cells. Broadly, the virulence factors of *S. aureus* can be classified into two groups-(a) Virulence factors involve in establishment of infection and (b) Virulence factors involve in maintenance of infection. The first step in establishing infection is the initial attachment of *S. aureus* to eukaryotic membrane followed by colonization and subsequent infection (Brouillette et al, 2003). Colonization is

commonly associated with a variety of adherence factors or adhesins which are known as microbial surface component recognizing adhesive matrix molecules (MSCRAMMs). These surface molecules attach to components of host tissue or plasma (Gordon and Lowy, 2008; Bien et al., 2011; Babra et al., 2013). There are over 20 different MSCRAMMs identified which can be expressed in *S. aureus* (Walsh et al., 2008) and they mediate attachment to surface proteins of host cells including collagen, elastin, fibrinogen, thrombospondin, fibronectin, bone sialoprotein and laminin (Foster and Hook, 1998). A number of MSCRAMMS take part in the formation of biofilm. Major adhesins in this group that mediate the initial attachment of bacteria to the bovine mammary gland, providing the first critical step for establishing infection (Foster and Hook, 1998) include FnBPA, FnBPB, ClfA, ClfB, CNA and BBP ([Cucarella, *et al.*, 2001, Gordon & Lowy, 2008, Plata, et al., 2009). On the other hand, the virulence factors/ antigens particularly in biofilm formation include PNAG, Bap, Protein A, Agr, SasC and SasG, eDNA etc. (Lasa and Penades, 2006; Corrigan et al., 2007; Merino et al., 2009; Schroeder et al., 2009; Boles and Horswill 2011; Archer et al., 2011).

Protein A is a crucial virulence factor of *S. aureus* which suppresses the host immune response because of its anti-opsonic and antiphagocytic property preventing clearance of around 99% of bacteria (Forsgren, 1970). Besides, Protein A contributes to the lack of immunity in host to succeeding *S. aureus* infections by exhibiting B-cell associated super antigenic property (Kim et al., 2012). FnBPs mediate interactions of *S. aureus* with fibrinogen and fibronectin leading to formation of biofilm (Greene et al., 1995). It also helps in invasion *S. aureus* into mammary epithelial cells. ClfA and ClfB mediate *S. aureus*-induced platelet aggregation through their fibrinogen binding function (O'Brien et al., 2002).

S. aureus produces a variety of virulence factors which permit the pathogen to evade the tissue and host immune system and thereby assisting in the maintenance of infection. These virulence factors include capsular polysaccharides, super antigenic exotoxins and cytotoxins. A large number of cytotoxins are produced by *S. aureus* which form pores in the cell membrane causing osmotic swelling leading to cell death. These cytotoxins include leukocidins, phenol soluble modulins (PSMs) and cytolysins. The cytolysins of *S. aureus* are α-, β-, γ-, and δ-toxins, of which α-toxin is

well characterised and most potent toxin (Wadstrom 1983). β-toxin is a sphingomyelinaseC which has been reported to be produced by 95% of the bovine mastitis isolates (Tollersrud, 2001). This toxin causes damage to epithelial lining of mammary gland. Gamma toxin is produced by coagulase positive *S. aureus* causes tissue damage by stimulating degranulation of neutrophils. Delta toxin is responsible for lysis of cells in different species (Hirsh et al., 2004). Leukocidins target the phagocytic cells and cause pore in the membrane of PMNs and interfere with the function of neutrophis (Rainard et al., 2003). Members of Leukocidin family are Panton Valentine Leukocidin (PVL), LukR-PV, LukM/FPV, LukE/D, γ-haemolysin and Leukocidin**.** Phenol soluble modulins (PSMs) are the peptides produced by *S. aureus* which are cytotoxic and proinflammatory. Recently PSMs have been reported to play a role in the formation of biofilm by *S. aureus* (Periasamy et al., 2012). *S. aureus* also produces a number of superantigens including enterotoxins (SEs), Toxic Shock Syndrome toxin (TSST) and exfoliative toxins (ETA and ETB). Enterotoxins of *S. aureus* include the classical enterotoxins A to E and the recently identified and characterised ones (SEG-SEU) [Dinges et al., 2000; Smyth et al., 2005]. These antigens are considered superantigens due to their ability to release cytokines in from both T cells and macrophages by binding to outer surface of MHC class II proteins and T cell receptors (Marrack and Kappler, 1990; Balaban and Rasooly, 2000; Ulrich, 2000; Schlievert, 2008). Variability in the prevalence of virulence factors in *S. aureus* may result in various levels of severity and forms of mastitis in cows (Ikawaty et al., 2010). No studies have been done to investigate about the virulence factors of *S. aureus* from bovine mastitis origin in Australia. The aim of this study was to screen the bovine mastitis-associated *S. aureus* isolates for the distribution of different virulent factors including MSCRAMMS, cytotoxins and superantigens by using conventional polymerase chain reaction (PCR) and the available serological methods.

4.2 Materials and methods

4.2.1 *S. aureus* isolates

One hundred and fifty four (154) fully characterised *S. aureus* strains of Australian origin isolated from clinical cases of mastitis in cows in Victoria and Queensland were used for the study. ATCC® 29213™, ATCC® 49775™, and ATCC® 8096™

were used as positive controls in the study. *S. aureus* strains representing CP types 1 (strain M), 2 (strain Smith diffuse), 5 (strain Newman), 8 (USA 400 MW2) and a non-capsulated strain (LAC, USA 300) were donated by Professor Gerald Pier (Harvard Medical School, Boston, USA) were used as positive controls. Besides BOAISRF *S. aureus* 25, 125 and human *S. aureus* 11 which were characterised in our laboratory were also used as positive controls in the study. These isolates were grown on Mueller Hinton (MH) agar and subcultured in nutrient broth supplemented with 1% glucose and stored on cryobeads (Blackaby Diagnostics) or as glycerol (15%) broth stocks at -80°C.

4.2.2 Detection of enterotoxins in *S. aureus* isolates

Detection of the SEA, SEB, SEC1, SEC2, SEC3, SED and SEE toxins in the 154 strains of *S. aureus* was carried out using 3M™ Tecra™ Staph Enterotoxins Visual Immunoassay or 3M™ Tecra™ SET VIA kits (3M Australia Pty Ltd). The test was performed according to the instructions of the manufacturer. Briefly, wash solution provided with the kit was used to soak *S. aureus* cells and incubated at room temperature. 200µL sample was added to the SE pre-coated wells and read optical density at 405 nm. Samples showing OD ≥0.2 were considered positive. Two sets of positive and negative controls provided with the kit were run along.

SET-RPLA Toxin Detection kit (Themo-Fisher Scientific, Australia) was used to confirm presence or absence of enterotoxin A-D in 154 *S. aureus* strains of bovine origin. Briefly, bacterial suspension prepared as per the protocol provided with the kit and latex particles linked to anti-enterotoxin A-D antibodies was added to bacterial suspension. The antigen-antibody suspensions were incubated at room temperature for 24 h and observed for agglutination to confirm the presence of enterotoxins.

4.2.3 Detection of *pvl* and *mecA* gene in *S.aureus* isolates

Detection of *mecA* and *pvl* genes was accomplished using the GenoType® MRSA assay (Hain-Lifesciences). The test is based on the DNA strip technology involving three steps viz. isolation of DNA from cultured material, multiplex PCR amplification with biotinylated primers and reverse hybridization.

4.2.4 Extraction of genomic DNA

Genomic DNA was extracted from 154 *S. aureus* isolates using a kit (MO BIO Laboratories, Inc Carlsbad, CA). The extracted genomic DNA was stored at -20°C until use.

4.2.5 PCR amplifications of different virulent genes of *S. aureus*

Conventional PCR was carried out to detect 32 different virulent genes of *S. aureus* isolated from clinical bovine mastitis cases in Australia. The primers, Tms, band size for all the genes are provided in Table 4.1. Following were the conditions used for detection of different virulence factors:

The amplification conditions for *tsst-1, clfA, clfB, cna* and *spa* are 95°C for 5 min, 30 cycles of 95°C for 30 sec, Tm for 30 sec and 72°C for 45 sec with a final extension of 72°C for 10min. The PCR conditions for *fnbpA, fnbpB, hlb, mecA, sdrE, bbp, isdA* and *sdrD* are 95°C for 5 min, 35 cycles of 95°C for 30 sec, Tm for 30 sec and 72°C for 45 sec with a final extension of 72°C for 10min. *isdB* primers were developed in our lab (Babra et al., 2014) with the amplification conditions at 35 cycles of 95°C for 30 sec, Tm for 1min and 72°C for 2 min with a final extension of 72°C for 10min. Amplication of *hla* worked better at 95°C for 5 min, 38 cycles of 95°C for 30 sec, Tm for 30 sec and 72°C for 45 sec with a final extension of 72°C for 10min. The amplification conditions for *sea, seb, sec, sed, see, seg, seh, sei* and *sej* were 95°C for 5 min, 30 cycles of 95°C for 2 min, Tm for 1 min and 72°C for 1 min with a final extension of 72°C for 5min. *eta* and *etb* were amplified at 95°C for 5 min, 30 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min with a final extension of 72°C for 10min.

All PCR products were analysed by agarose gel (1.5%) electrophoresis, staining with 0.8uL/100mL of Midori Green DNA Stain (Nippon Genetics) using 1x SB Buffer. An O'RangeRuler DNA Ladder (100-1500 bp, Fermentas) was used for comparing the approximate band sizes after visualising on UV transilluminator.

Table 4.1: PCR amplification of virulence factor genes of *S. aureus* isolates from bovine mastitis

Virulence factor gene	Positive control	Primer Forward (5'-3')	Primer Reverse (5'-3')	Tm	Expected band size (bp)	Reference
cna	Strain M (CP1)	AAA GCG TTG CCT AGT GGA GA	AGT GCC TTC CCA AAC CTT TT	50°C	192	Smith et al., 2010
clfa	Strain M (CP1)	CGC CGG TAA CTG GTG AAG CT	TGC TCT CAT TCT AGG CGC ACT T	55°C	314	Stutz et al., 2011
clfb	Strain M (CP1)	ATG ATC TTG CTT GCG TT	CCG ATT CAA GAG TTA CAC C	47°C	215	Stutz et al., 2011
spa	ATCC 29213	TCA AGC ACC AAA AGA GGA AGA	GTT TAA CGA CAT GTA CTC CGT TG	51°C	Variable	Montesinos et al., 2002
fnbpA	LAC USA 300 (CP neg)	GCG GAG ATC AAA GAC AA	CCA TCT ATA GCT GTG TGG	48°C	1279	Booth et al., 2001
fnbpB	LAC USA 300 (CP neg)	GGA GAA GGA ATT AAG GCG	GCC GTC GCC TTG AGC GT	56°C	820	Booth et al., 2001
bbp	Strain M (CP1)	AAC TAC ATC TAG TAC TCA ACA ACA G	ATG TGC TTG AAT AAC ACC ATC ATC T	53°C	575	Tristan et al., 2003
isdA	ATCC 29213	CTG CGT CAG CTA ATG TAG GA	TGG CTC TTC AGA GAA GTC AC	52°C	332	Verkaik et al., 2010
isdB	BOAISRF S. aureus 25	ACG AGA GTT TGG TGC GCT AT	GTT GAG GCC CCT ACT TCT GA	55°C	192	Babra et al., 2014
sdrD	Strain Newman (CP5)	CGG AGC TGG TCA AGA AGT AT	TGC CAT CTG CGT CTG TTG TA	52.3° C	500	Verkaik et al., 2010
sdrE	Strain Newman (CP5)	AGA AAG TAT ACT GTA GGA ACT G	GAT GGT TTT GTA GTT ACA TCG T	50°C	433	Sabat et al., 2006
tsst-1	LAC USA 300 (CP neg)	ACC CCT GTT CCC TTA TCA TC	TTT TCA GTA TTT GTA ACG CC	53°C	326	Nada et al., 2012
eta	Hu S. aureus 11	GCA GGT GTT GAT TTA GCA TT	AGA TGT CCC TAT TTT TGC TG	58°C	93	Sauer et al., 2008
etb	Hu S. aureus 11	ACA AGC AAA AGA ATA CAG CG	GTT TTT GGC TGC TTC TCT TG	58°C	226	Sauer et al., 2008
hla	USA 400 MW2 (CP8)	GTA CTA CAG ATA TTG GAA GC	GTA ATC AGA TAT TTG AGC TAC	47°C	274	Ramakrishna et al., 2013
hlb	USA 400 MW2 (CP8)	GCC AAA GCC GAA TCT AAG	CGC ATA TAC ATC CCA TGG C	51°C	840	Booth et al., 2001
sea	Smith Diffuse (CP2)	TTG GAA ACG GTT AAA ACG AA	GAA CCT TCC CAT CAA AAA CA	50°C	120	Rall et al., 2008
seb	Smith Diffuse (CP2)	TCG CAT CAA ACT GAC AAA CG	GCA GGT ACT CTA TAA GTG CC	50°C	478	Rall et al., 2008
sec	Smith Diffuse (CP2)	GAC ATA AAA GCT AGG AAT TT	AAA TCG GAT TAA CAT TATA CC	50°C	257	Rall et al., 2008
sed	ATCC 8096	CTA GTT TGG TAA TAT CTC CT	TAA TGC TAT ATC TTA TAG GG	50°C	317	Rall et al., 2008
see	ATCC 8096	AGG TTT TTT CAC AGG TCA TCC	CTT TTT TTT CTT CGG TCA ATC	50°C	209	Rall et al., 2008
seg	Smith Diffuse (CP2)	AAG TAG ACA TTT TTG GCG TTC C	AGA ACC ATC AAA CTC GTA TAG C	55°C	287	Rall et al., 2008
seh	Smith Diffuse (CP2)	GTC TAT ATG GAG GTA CAA CAC T	GAC CTT TAC TTA TTT CGC TGT C	48.4° C	213	Rall et al., 2008
sei	Smith Diffuse (CP2)	GGT GAT ATT GGT GTA GGT AAC	ATC CAT ATT CTT TGC CTT TAC CAG	50°C	454	Rall et al., 2008
sej	BOAISRF S. aureus 125	CAT CAG AAC TGT TGT TCC GCT AG	TGA ATT TTA CCA TCA AAG GTA C	50°C	142	Rall et al., 2008

4.3 Results

Both the conventional PCR and serological methods of detection, used in this study revealed that α-toxin (94.16%) and β-toxin (83.12%) are the predominant cytotoxins detected in *S.aureus* isolated from clinical bovine mastitis cases in Australia. Among the superantigens *seh* (32.5%), *sec* (23.4%), *seg* (17.5%) and *sei* (13%) were the more prevalent enterotoxins in bovine *S.aureus* isolates. Details of prevalence and/or distribution of cytotoxins and superantigenic toxins among the bovine *S. aureus* strains are presented in Table 4.2 and Figure 4.1.

The study revealed that *clfA* (91.56%), *clfB* (92.86%), *spa* (87.7%), *fnbpA* (54.5%), *isdA* (98.1%), *isdB* (100%), *sdrD* (98.1%) and *sdrE* (95.5%) are the predominant MSCRAMMS present in the *S. aureus* isolates. None of the strains are found to be positive for *mecA*.The detail finding of MSCRAMMS in bovine *S. aureus* isolates is presented in table 4.3 and Figure 4.2.

Table 4.2: Distribution of toxin genes in *S. aureus* isolates of bovine mastitis origin in Australia

***S. aureus* isolates**	***hla***	***hlb***	***tsst-1***	***eta***	***etb***	***pvl***	***sea***	***seb***	***sec***	***sed***	***see***	***seg***	***seh***	***sei***	***sej***
154	145	128	13	0	0	4	4	1	36	2	1	27	50	20	2
percentage	94.16	83.12	8.44	0	0	2.6	2.6	0.65	23.4	1.3	0.65	17.5	32.5	13	1.3

Figure 4.1: Comparison of distribution of the genes encoding toxins in *S. aureus* isolated from bovine mastitis cases in Australia

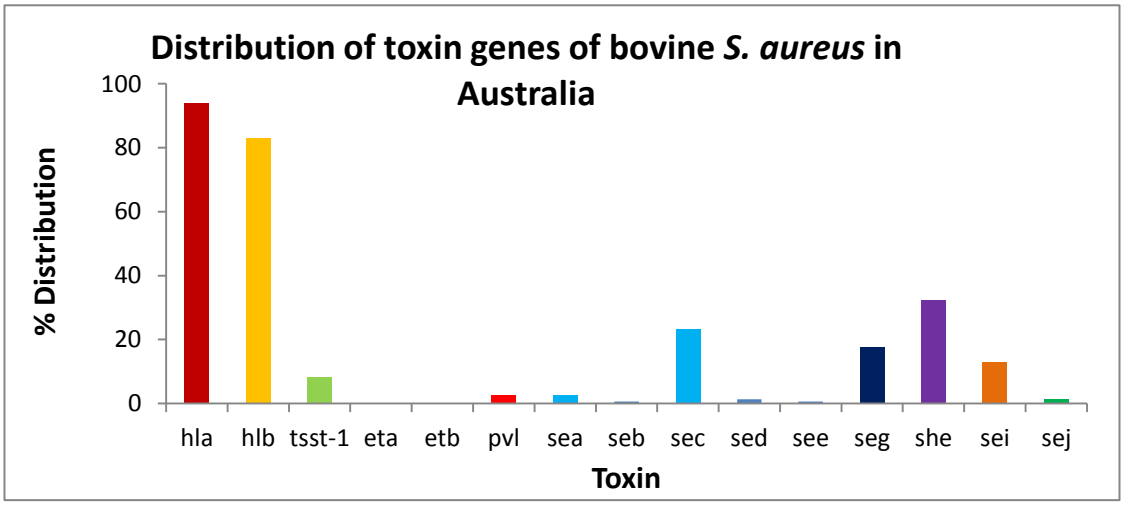


Table 4.3: Distribution of MSCRAMMS genes in *S. aureus* isolates of bovine mastitis origin in Australia

***S. aureus* isolates**	***cna***	***clfA***	***clfB***	***spa***	***fnbpA***	***fnbpB***	***bbp***	***isdA***	***isdB***	***sdrD***	***sdrE***
154	48	141	143	135	84	2	14	151	154	151	147
Percent positive	31.2	91.56	92.86	87.7	54.5	1.3	9.09	98.1	100	98.1	95.5

Figure 4.2: Comparison of distribution of MSCRAMMS genes among *S. aureus* isolates of bovine mastitis origin

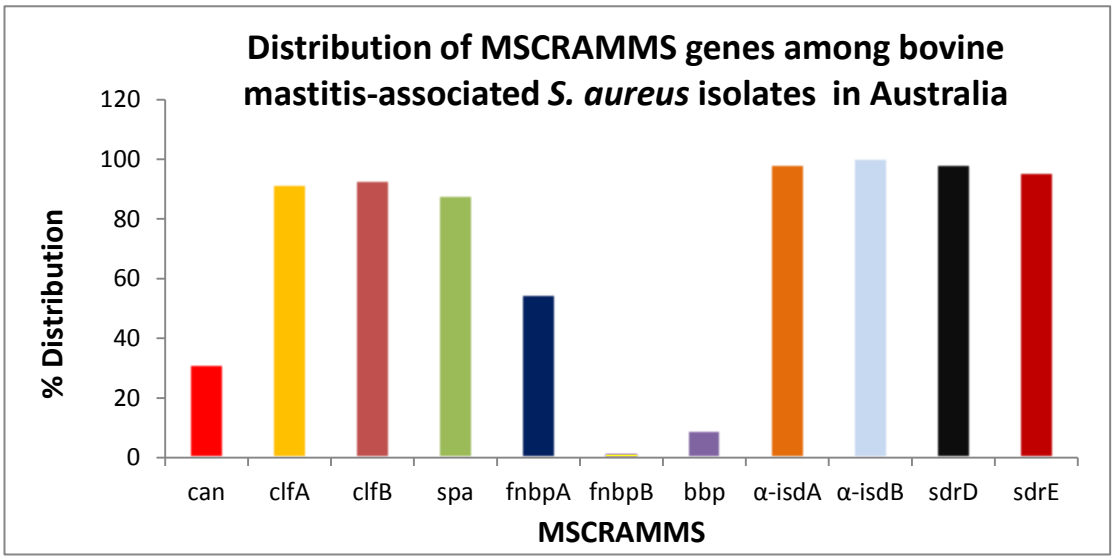
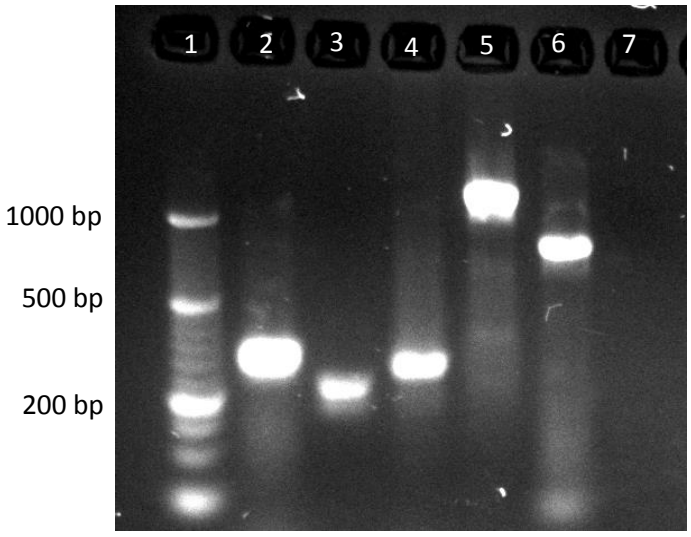


Figure 4.3: Amplification of *clfA, clfB, spa, fnbpA* and *fnbpB* using conventional PCR



Lane 1– 50 bp DNA ladder

Lane 2 – *clfA*, 192 bp

Lane 3 – *clfB*, 215 bp

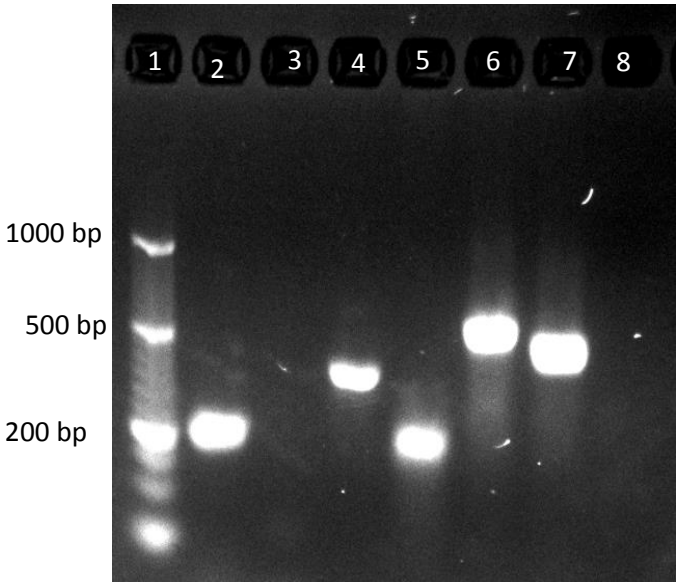
Lane 4 – *spa* 300 bp

Lane 5– *fnbp*A, 1279 bp

Lane 6 – *fnbpB*, 820 bp

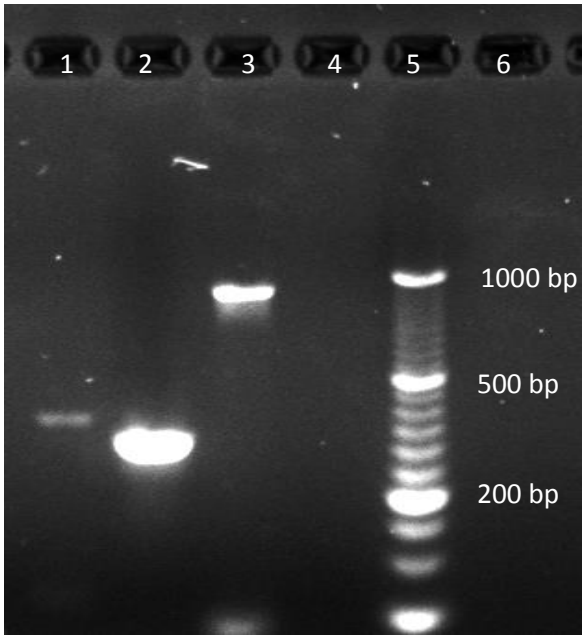
Lane 7– Negative control

Figure 4.4: Amplification of *cna*, *isdA*, *isdB*, *sdrD* and *sdrE* in PCR



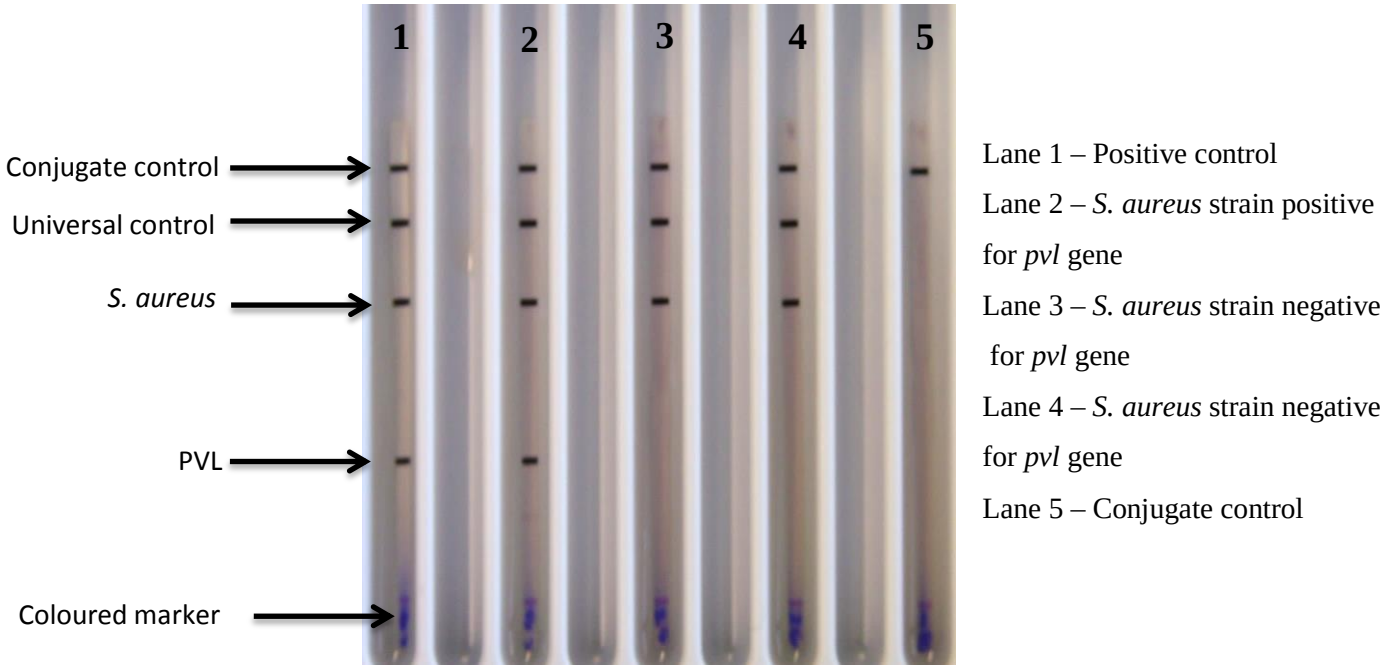
Lane 1 – 50 bp DNA ladder
Lane 2 – *cna*, 192 bp
Lane 4 – *isdA*, 332 bp
Lane 5 – *isdB*, 192 bp
Lane 6 – *sdr*D, 500 bp
Lane 7 – *sdrE*, 433 bp
Lane 8 – Negative control

Figure 4.5: Amplification of *tsst-1*, *hla* and *hlb* in PCR



Lane 1 – *tsst-1*, 326 bp
Lane 2 – *hla*, 274bp
Lane 3 – *hlb*, 840 bp
Lane 4 – Negative control
Lane 5 – 50 bp DNA ladder

Figure 4.6: Detection of PVL gene by using GenoType® MRSA kit



Conjugate control: documenting the efficiency of conjugate binding and Substrate reaction, **Universal control:** It indicates the presence of bacterial DNA and the correct implementation of DNA isolation and amplification, ***S. aureus:*** 85 bp *S. aureus*-specific gene, **PVL:** 77 bp PVL fragment

4.4 Discussion

This study was conducted to determine the distribution of virulence factors produced by *S. aureus* isolated from clinical bovine mastitis cases in Australia. Due to emergence of resistance against antibiotics, virulence factors of *S. aureus* including MSCRAMMS and exoproteins have been potential targets to develop a suitable vaccine against bovine mastitis. Various investigators have suggested incorporation of predominant virulence factors as potential vaccine candidates while formulating a multicomponent vaccine as they have ability to trigger immune response (Verkaik et al., 2010). The current study has highlighted that *clfA* (91.56%), *clfB* (92.86%), *spa* (87.7%), *fnbpA* (54.5%), *isdA* (98.1%), *isdB* (100%), *sdrD* (98.1%), *sdrE* (95.5%) are the predominant MSCRAMMS detected and/or produced by *S. aureus* of clinical bovine mastitis origin. This finding is in accordance with the findings in other countries (Karahan et al., 2011; Zhao et al., 2011; Sorum et al., 2013). The predominant toxins in the *S. aureus* isolates in the present study are α-toxin

(94.16%), β-toxin (83.12%) which is in accordance with the finding of Ikawaty et al (2011) in Netherlands.

Among the superantigens, the predominant toxins, in descending order were *seh* (32.5%), *sec* (23.4%), *seg* (17.5%) and *sei* (13%), respectively, with the *sea*, *seb*, *sed*, *see*, *sej* present in a low numbers ranging from 0.65-2.6%. All the previous investigations for detection of *pvl* gene in *S. aureus* isolates from bovine mastitis origin have pointed towards the results being negative (Prashanth et al., 2011; Gindonis et al., 2013; Pu et al., 2014) except one finding by Yamada et al (2005) who reported presence of pvl gene in 62.5% of *S. aureus* isolates from cases of bovine mastitis in Japan. In our study, 4 strains (2.6%) of *S. aureus* were found to be positive for *pvl* gene. The PVL toxin is responsible for destruction of leukocytes and necrosis of mammary tissue in bovine mastitis.

None of the experimental vaccines or the available commercial vaccines has been demonstrated to prevent new intra-mammary infection (IMI), although some have been reported to reduce the clinical severity of the mastitis. MSCRAMMS including ClfA, ClfB, IsdB, FnBP, SdrD, SdrE (Stranger-Jones et al., 2006; Maira Litran et al., 2005; Middleton, 2008) and toxins including TSST-1, SEC, β toxin and α toxin (Spaulding et al., 2012) have demonstrated partial protection against *S. aureus* infections in mice when used as single component vaccines. Perhaps the answer to the difficulty being faced in the development of an effective vaccine may lie in the identification of the MSCRAMMs expressed, not just detected, *in vivo* in the mammary gland following infection, which studies warrant urgent attention.

Despite the potential of the exotoxins and cytotoxins to inflict damage to the mammary gland epithelial cell lining (Ikawaty et al., 2010 and the variability in the distribution of clinical *S. aureus* isolates, it may be merit worthy to confine the development of vaccines against *S. aureus* infections to the predominant MSCRAMMS such as ClfA, ClfB, ProteinA, isdA, isdB, SdrD and SdrE and toxins such as α toxin and β toxin as potential vaccine candidates against bovine mastitis caused by *S. aureus*.

Chapter 5: Serological versus molecular typing of *Staphylococcus aureus* isolated from clinical and subclinical cases of bovine mastitis in Australia

5.1 Introduction

One of the important virulence factors in variety of staphylococcal infections is the capsule, which constitutes an integral part of cell wall (Thakker et al., 1998). Capsular polysaccharide (CP) protects the bacterial cell from phagocytosis (Verdier et al., 2007) and has been reported to promote prolonged persistence of the bacteria in blood of host thus enhancing microbial virulence (O'Riordan and Lee, 2004).

Karakawa and Vann in 1982 reported for the first time that most *S. aureus* isolates are encapsulated and identified 8 different capsular serotypes. Guidry et al (1998) reported that *S. aureus* isolated from human infections consisted of 12 polysaccharide serotypes, 11 different capsular serotypes and one non-capsular surface polysaccharide 336. The majority of clinical isolates were reported to express a surface polysaccharide of either serotype 5 or 8, representing 70-80% of the isolates from all sources (Arbeit et al., 1984; Hochkeppel et al., 1987; Poutrel et al., 1988). However, the percentage distribution of these capsular types appears to vary in different countries (Arbeit et al., 1984; Hochkeppel et al., 1987; Poutrel et al., 1988; Roghmann et al., 2005; Han et al., 2000; Sompolinsky et al., 1985). Lee (2002) suggested that there were only four types of capsular polysaccharides, all other reported types representing mutated versions which still need elucidation. The rest were designated non-typeable *S. aureus*. Majority of these strains were reported to possess a unique surface polysaccharide antigen 336 (Guidry et al., 1998), which contained polyribitol-phosphate-*N*-acetylglucosamine, a component of cell wall teichoic acid (Verdier et al., 2007).

Several studies have shown that purified capsular polysaccharides are poor immunogens, when used alone (O'Riordan and Lee, 2004; Jones, 2005), in eliciting protective immune responses, potentially because of T-cell independence and homo-polymeric structure. However, upon cross-linking to high molecular weight carrier proteins, immunogenicity is improved due to the induction of T-cell-dependent

immune responses. For the designing CP-based vaccines, knowledge of the prevalence and type of CP is essential (Ma et al., 2004). Since the prevalence of CPs may be different in different countries (Arbeit et al., 1984; Sompolinsky et al., 1985; Poutrel et al., 1988; Han et al., 2000; Roghman et al., 2005), either specific formulations may be needed to control mastitis caused by *S. aureus* in different regions of the world or a conjugate vaccine formulation containing all the capsular types prevalent in different countries that is capable of producing antibodies against CP antigen also is needed. Capsular polysaccharide of *S. aureus* being the important immune evasion molecule (Nanra et al., 2013) has been used in the development of conjugate vaccine candidates. Since numerous capsular types of *S. aureus* exist around the world, it is important to know their prevalence in Australia in order to rationally select suitable conjugate vaccines in Australia for the prevention of bovine mastitis. However, there are no reports on the prevalence of different CP types of *S. aureus* causing bovine mastitis in Australia. Hence the objective of this study was to determine the distribution of different capsular phenotypes among the Australian *S. aureus* strains isolated from clinical bovine mastitis cases.

Detection of capsule has been done by using a variety of methods such as characterisation of colony morphology in serum-soft agar, absence of clumping factor, negative staining by using nigrosin. These methods are considered crude and few *S. aureus* strains can be detected for capsule (O'Riordan and Lee, 2004). The present study was undertaken to investigate the prevalence of capsular types among the bovine mastitis-associated *S. aureus* isolates in Australia by using both serological and genotyping methods with one of the traditional methods that is negative staining.

The main objectives of the study were to (a) produce CP-specific and surface polysaccharide-336 specific sera in mice that would not cross react with the other serotypes (b) identify the antibody isotypes produced in the sera (c) detection of presence or absence of capsule by using specific capsular stain (d) genotyping of the Australian *S. aureus* isolates using PCR (e) serologically type the *S. aureus* isolates of Australian origin by using CP-specific antisera.

5.2 Material and Methods

5.2.1 Animal ethics approval

A total of 180 six week-old specific pathogen-free Quackenbush Swiss line 5 mice (30 mice per capsular type including control) were obtained from the Animal Resources Centre, Perth, Western Australia and used for production of CP and antigen 336-specific antisera. All animal experiments were carried out with the approval of Curtin University's Animal Ethics Committee (Approval No: AEC_2011_65) ensuring compliance with the Western Australian Animal Welfare Act, 2002.

5.2.2 Capsular and surface polysaccharide specific *S. aureus* strains used in the study

One hundred and fifty four (154) fully characterised *S. aureus* strains of Australian origin isolated from clinical cases of mastitis in cows in Victoria and Queensland were used for the study. Reference strains representing CP types 1 (strain M), 2 (strain Smith diffuse), 5 (strain Newman), 8 (USA 400 MW2) and a non-capsulated strain (LAC, USA 300) were donated by Professor Gerald Pier (Harvard Medical School, Boston, USA). *S. aureus* ATCC-55804 designated as serotype 336 was purchased from ATCC, USA. These isolates were grown on Mueller Hinton (MH) agar and subcultured in nutrient broth supplemented with 1% glucose and stored on cryobeads (Blackaby Diagnostics) or as glycerol (15%) broth stocks at -80°C.

5.2.3 Production of antisera for serological typing

Preparation of the different anti-CP type 1, 2, 5, 8 and SP-336 antisera was carried out according to Fournier et al. (1984). Briefly, *S. aureus* CP types-1, 2, 5, 8 and SP-336 were grown on MH agar at 37^{o}C for 18 h. Plates containing colonies were washed with 20 ml of 3% formalinised PBS. The formalin-killed bacterial suspension was allowed to remain at room temperature for a period of 24 h followed by centrifugation at 10,000 x g for 20 min and washing with PBS five times. The pellet was resuspended in sterile PBS, sterility confirmed by absence of growth on MH agar and then stored at 4^{o}C. Mice were immunized every week for 5 weeks with formalin-killed *S. aureus*. The first three doses contained the equivalent to $5x10^{7}$, $1x10^{8}$ and $5x10^{8}$ CFU, respectively, and were administered intraperitoneally in 0.2

mL volume. For the fourth and fifth doses, the bacterin was mixed with equal proportion with Imject Alum (Thermo Scientific) and 0.2 mL containing an equivalent of $1x10^9$ and $5x10^9$ CFU, respectively, were administered subcutaneously one week apart. The control mice were vaccinated with PBS by using the same routes. Mice were bled and sera were obtained. The CP-/SP-specific antisera were cross-absorbed with non-capsulated *S. aureus* strain LAC, USA 300, followed by cross-absorption with the heterologous CP types CP1, CP2, CP5, and CP8 depending upon the desired CP specificity. For SP-336 serum, the same method was followed, initially cross absorbed with non-capsulated LAC USA 300 followed by cross absorption with CP 1, 2 5 and 8.

5.2.3.1 Detection of antigen specific antibody isotypes in sera samples

Antigen-specific antibody isotypes determination was carried out on both immunised and control sera samples. The levels of anti- *S. aureus* IgA, IgM, IgG, IgG_1, IgG_{2a} and IgG_{2b} were determined by using an indirect ELISA (Fry, 2006). Formalinised killed *S. aureus* suspended in PBS representing different CP types was used as coating antigens. Conjugated antibodies used were affinity-purified anti-mouse heavy chain goat IgA, IgM, IgG, IgG_1, IgG_{2a} and IgG_{2b} conjugated with alkaline phosphatase. The enzyme substrate was p-nitrophenyl-phosphate and absorbance at 405 nm was measured with a micro-plate reader. Briefly, Nunc-Immuno™ MicroWell™ 96 well solid plates (Sigma Aldrich Pty Ltd, Australia) were coated with 100µl of CP8 positive, CP5 positive, CP2 positive, CP1 positive and SP-336 positive *S. aureus* (OD adjusted to 0.3 at 630 nm) and incubated overnight at 4ºC. Wells were washed using wash buffer (Tris gelatine-0.05% Tween 20, pH 7.4) and blocked with blocking buffer (Tris/gelatin/BSA, pH 7.4) at 37 ºC for 2 h followed by washing of the wells thrice and adding 100µl of 2-fold serial dilutions of test and negative control sera in triplicates. Plates were then incubated at 37ºC for 2 h followed by rewashing of the wells using wash buffer. An aliquot of 100µl of 1: 10,000 dilution of alkaline phosphatase labelled goat antimouse IgA, IgM, IgG, IgG_1, IgG_{2a} or IgG_{2b} were added to the wells and incubated at 4ºC overnight. After washing for three times, substrate was added and the plates were read at 405 nm using ELISA plate reader (Model 550, Borad). Specific absorbance versus serum dilutions were plotted to calculate antibody titres.

5.2.4 CP serotyping of *S. aureus* isolates

Slide agglutination test was performed to determine the serotype of the strains as described elsewhere (Verdier et al., 2007). Briefly each strain was grown overnight on MH agar and a single colony was resuspended in a drop of 0.9% normal saline in a clean glass slide. A drop of serum was added and mixed with the culture using a tooth pick and observed for agglutination in less than 20 sec. The strains, which showed no agglutination against any of the CP-specific antisera, were further tested for agglutination using anti-SP336 serum. Strains found negative with all the five specific sera were considered as non-typeable.

5.2.5 CP genotyping of *S. aureus* isolates

Genomic DNA was extracted from *S. aureus* using a kit (MO-Bio or Real Biotech). Detection of the presence of *cap*1, *cap*2, *cap*5 and *cap*8 loci in all the strains was carried out by Polymerase Chain Reaction (PCR). The primers used for typing *cap*1 are 5'-AGG TCT GCT AAT TAG TGC AA-3' (forward) and 5'-GAA CCC AGT ACA GGT ATC ACC A-3' (reverse) with an expected product size of 550 bp and for *cap*2 are 5'-AGC AAT CTT CGG TTA TTG CCG GTG-3' (forward) and 5'-ATG ACG GTA AGG CAT CAA GGT CG-3' (reverse) with an expected amplicon size (non-specific). The PCR cycling parameters for both *cap*1 and *cap*2 were: denaturation at 94^{o}C for 5 min followed by 94^{o}C for 30 sec, Tm at 57^{o}C (*cap*1) or 60^{o}C (*cap*2) for 30 sec, 72^{o}C for 60 sec with 25-30 cycles and final extension at 72^{o}C for 5 min.

The primers used for typing *cap*5 and *cap*8 were 5'- ATG ACG ATG AGG ATA GCG-3' (forward) and 5'- CTC GGA TAA CAC CTG TTG C-3' (reverse) for *cap*5 and 5'- ATG ACG ATG AGG ATA GCG-3' (forward) and 5'- CAC CTA ACA TAA GGC AAG-3' (reverse) for *cap*8, respectively (Moore et al., 2001). The expected band sizes were 881 bp and 1148 bp for *cap*5 and *cap*8, respectively. Thermal cycling conditions were denaturation at 95^{o}C for 5 min, 95^{o}C for 30 sec, Tm of 55^{o}C (*cap*5) or 52^{o}C (*cap*8) for 30 sec, 72^{o}C for 5 min with 25-30 cycles and the final extension at 72^{o}C for 5 min.

The PCR products were analysed by agarose gel (1.5%) electrophoresis, Midori Green staining and UV trans-illumination. The positive controls included strains M,

Smith Diffuse, Newman and USA 400 for *cap*1, *cap*2, *cap*5 and *cap*8, respectively, and LAC, USA 300 was used as negative control.

5.2.6 Microscopic detection of capsule by using negative staining

Negative staining using nigrosin was performed on all the six strains of *S. aureus.* One drop of nigrosin was placed on a clean grease free slide; to which a few colonies of *S. aureus* were mixed avoiding the spread of the drop. Using another clean glass slide a thin smear was prepared and the film was allowed to air dry. The slide was saturated with crystal violet for one minute followed by rinsing with tap water. The slide was allowed to air dry before observing the smear under oil immersion. *S. aureus* stained purple with dark background and the capsule appeared clear (Figure 5.5A, B).

5.2.7 Statistical analysis

Correlation coefficients, represented as Pearson *r* values, between the serological with the genotyping method, for CP1, CP5, CP8 positive and non-typeable *S. aureus* strains, were determined using Microsoft Excel, Windows 10.

5.3 Results

5.3.1 Serotyping of capsular or antigen 336 in bovine *S. aureus* isolates

Serotyping of 154 *S. aureus* isolates using CP-specific sera (Table 5.1) confirmed the genotyping results; 36 (23.38%) and 49 (31.82%) of the isolates revealed the presence of CP5 and CP8, respectively, whereas none was positive for CP1. However, 14 (9.09%) strains were positive for CP2. The strains that were not agglutinated by any of the CP-specific sera were subjected to slide agglutination using anti- SP336 antiserum. Nine (5.84%) isolates were positive for SP-336 and the remaining 46 (29.87%) were declared as non-typeable (Table 5.1, Figure 5.3A).

5.3.2 Genotyping of capsular or antigen 336 in bovine *S. aureus* isolates

Genotyping of 154 *S. aureus* isolates revealed that 41 (26.62%) and 50 (32.47%) strains were positive for *cap*5 and *cap*8 (Table 5.1). None of the isolates were positive for the *cap*1 locus. The primers for the *cap*2 locus exhibited cross-reactivity with all the other three CP types producing amplicons of 700-800 bp (data not

shown). A total of 63 (40.91%) *S. aureus* strains, which carried none of the three loci (*cap*5, *cap*8 or *cap*1), were declared as negative by PCR (Table 5.1, Figure 5.3B).

5.3.3 Detection of capsule in *S. aureus* isolates by using negative staining

The negative staining using nigrosin corroborated the serological result. All the 99 *S. aureus* strains found positive in serology demonstrated capsule in negative staining method. The *S. aureus* with SP-336 though express surface polysaccharide but do not express any capsule (Verdier et al., 2007). The diagram of capsulated and non-capsulated *S. aureus* is presented in Figure 5.5 (A, B). However, an interesting result could be observed in detection of capsule by negative staining. Out of the 55 non-typeable strains confirmed by serological method, 13 *S. aureus* strains showed capsule while negative staining was performed. So, a total of 112 (72.72%) *S. aureus* strains demonstrated capsule using capsular staining method and 42 (27.27%) strains showed absence of capsule.

5.3.4 Analysis of antibody isotypes of pooled CP-specific antisera

The antibody isotypes in the 5 different sera revealed a diverse distribution of antibody activity. However, the predominant antibody isotypes in CP-8, 5, 2, 1 and SP-336 specific antisera were IgA, IgG2a and IgG, IgG_{2b} being present in only CP5-specific antisera. The detailed results are presented in Figure 5.1 and 5.2.

5.3.5 Statistical analysis

The correlation coefficient (*r*) between the serological and genotyping methods for detection of CP types 1, 5 and 8, and the non-typeable *S. aureus* isolates, was determined to be 0.97.

5.4 Discussion

Given the role of surface-associated polysaccharides in the virulence of *S. aureus*, epidemiological investigations on the distribution of capsular and surface polysaccharide types among *S. aureus* isolates is important for rational design of a vaccine formulation against infection with *S. aureus* regardless of their antibiotic susceptibility profiles. Numerous studies on human *S. aureus* capsular types have reported that 75-80% of all the isolates produce either CP5 or CP8 (Karakawa and

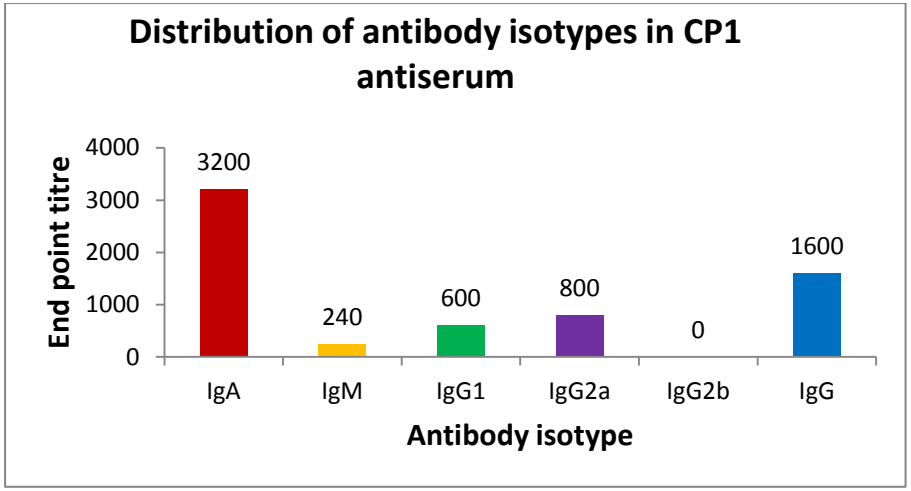
Van, 1982; Lee et al., 1990; Roghman et al., 2005). However, the prevalence of the CP serotypes (CP5 and 8) among the *S. aureus* strains isolated from cow's milk vary as low as 14% to as high as 95% (Poutrel et al., 1988; Guidry et al., 1998; Sordelli et al., 2000; Tollersrud et al., 2000). Paul-Satyaseela et al (2004) reported that CP typing was superior to bacteriophage typing whereas Schlichting et al (1993) had reported the capsular typing was less sensitive than genome typing yielding 26 different *S. aureus* types. However, neither serotyping for the capsular types 1 and 2 nor the localization of the potential antigens encoded by 26 types was determined. This study revealed that 64.29% of Australian bovine mastitis-associated *S. aureus* strains expressed capsule, of which, CP8 was predominant (31.82%), followed closely by CP5 (23.38%). In addition, 5.84% (nine of 154) of the isolates were positive for SP-336 and 30% of the isolates were non-typeable. Detection of 13 non-typeable but capsulated *S. aureus* strains using negative staining method is an important discovery of this study which indicated that there may be more than the 5 recognised types of CP and SP (4 CP types and 1 SP type) in *S. aureus* as proposed originally by Karakawa and Vann (1982). However, further studies for confirmation of this result are warranted.

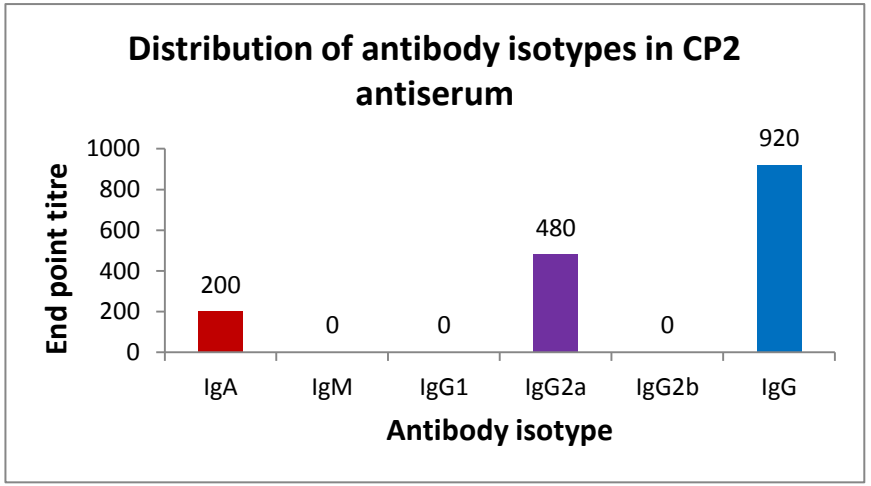
Very few studies have compared the performance of genotyping versus serology in the typing of *S. aureus* isolates. Most of the studies reported low proportion of phenotypic expression in comparison to genotypic expression of capsular polysaccharide types (Tollersrud et al., 2000; Camussone et al., 2012). In contrast, one study with human isolates, there was 100% correlation between capsular genotypes and phenotypes for CP5 and CP8 (Sutter et al., 2011). In this study, five (3.25%) bovine mastitis-associated strains carrying *cap*5 and one strain (0.65%) carrying *cap*8 were negative by agglutination. The discordance between genotyping and phenotyping may be attributable to non-expression of respective capsule-encoding genes, possibly due to mutations (Cocchiaro et al., 2006) or due to the difference in culture conditions *in vivo* and *in vitro* (Poutrel et al., 1995; Poutrel et al., 1997).

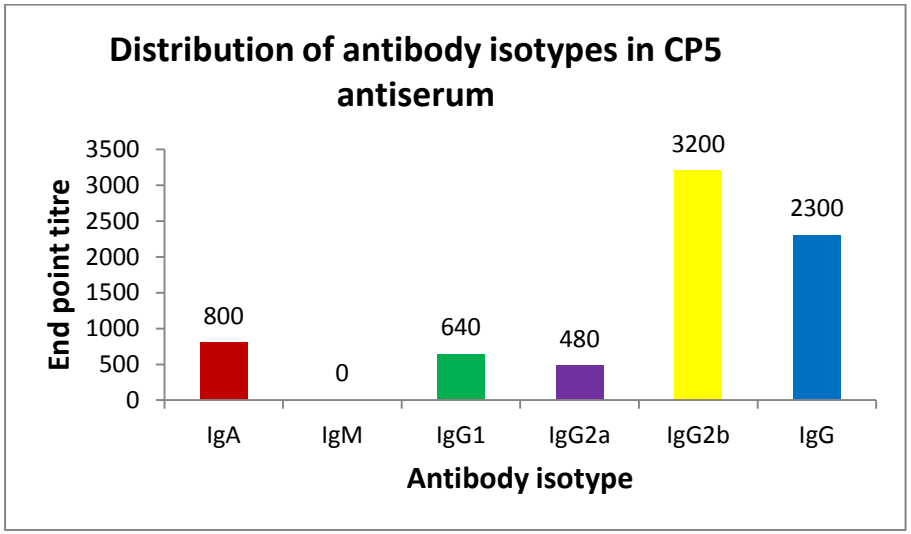
It is thus clear from this study that any surface-associated polysaccharide antigens-based vaccine formulation should not only include CP5 and CP8 types but also other capsular types with or without antigen 336 or poly-N-acetyl glucosamine (PNAG)

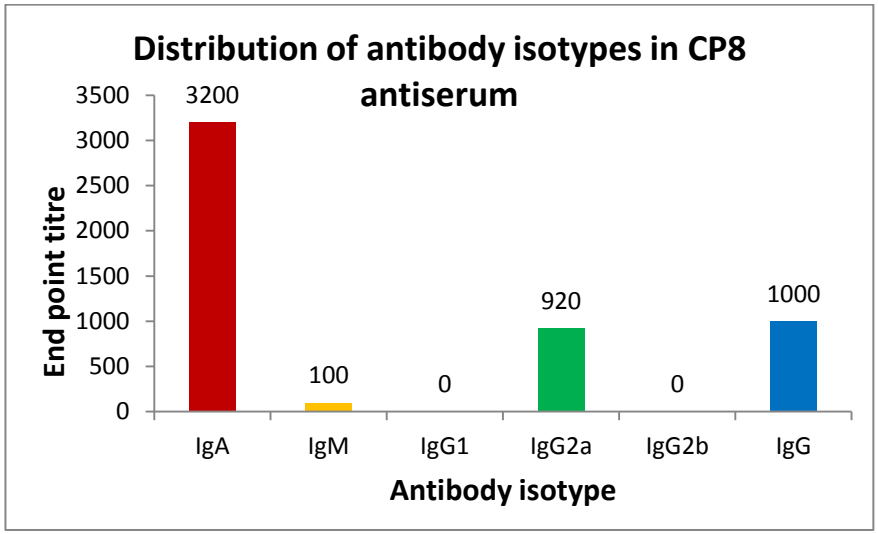
reported to be present in all *S. aureus* isolates (Gening et al., 2010) for prevention of clinical bovine mastitis caused by *S. aureus*. The prevalence of 29.87% of non-typeable *S. aureus* strains in Australia also highlights the need to explore the existence of other surface-associated polysaccharides including additional capsular phenotypes as proposed originally by Karakawa and Vann (1982).

Figure 5.1: Relative distribution of antibody isotype titres of CP and SP-336 specific antisera









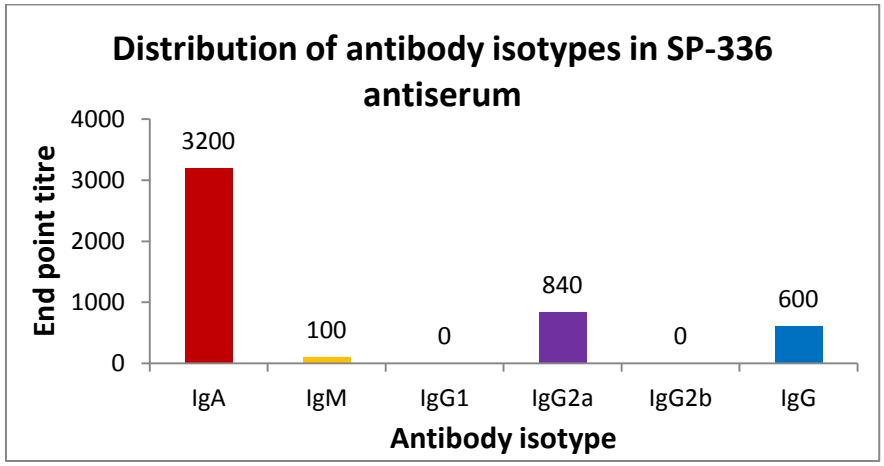


Figure 5.2: Comparative distribution of antibody isotype titres of CP and SP-336 specific antisera

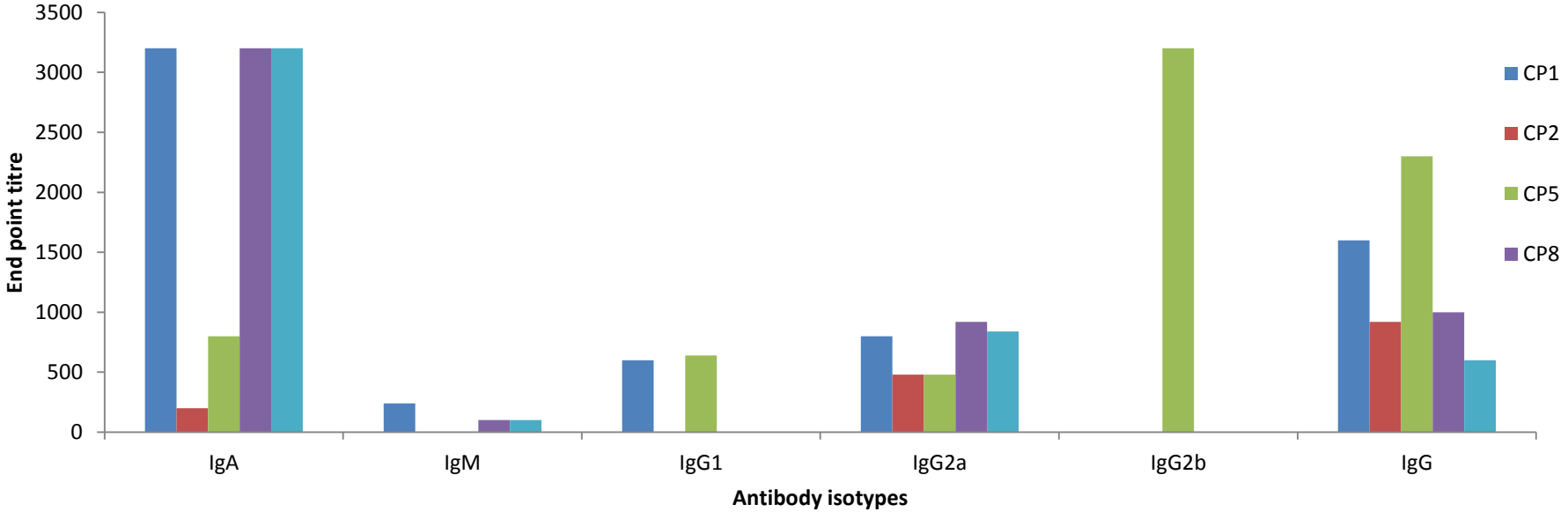


Table 5.1: Prevalence of capsular/surface polysaccharide genotypes and phenotypes in *S. aureus* isolates from cases of bovine mastitis in Australia

No (%) of *S aureus* isolates														
Total no. of isolates	**Detection of capsular type by slide agglutination test (SAT[a])**						**Detection of capsular type by PCR**						**Detection of capsule by staining**	
	CP1	**CP2**	**CP5**	**CP8**	**SP-336**	**NT[1]**	**CP1**	**CP2**	**CP5**	**CP8**	**SP-336**	**NT**	**Capsule +ve**	**Capsule -ve**
154	0	14 (9.09)	36 (23.38)	49 (31.82)	9 (5.84)	46 (29.87)	0	PDW[2]	41 (26.62)	50 (32.47)	PNA[3]	63 (40.91)	112 (72.73)	42 (27.27)

SAT[a]= Slide agglutination test NT[1]= Non typeable, PDW2= Primer did not work, PNA= Primer not available

Correlation coefficient (*r*) between SAT and PCR method for detection of CP1, CP5, CP8 and non-typeable *S. aureus* strains is 0.97.

Figure 5.3: Comparison of detection of CP/SP types in *S. aureus* by genotyping and serotyping methods

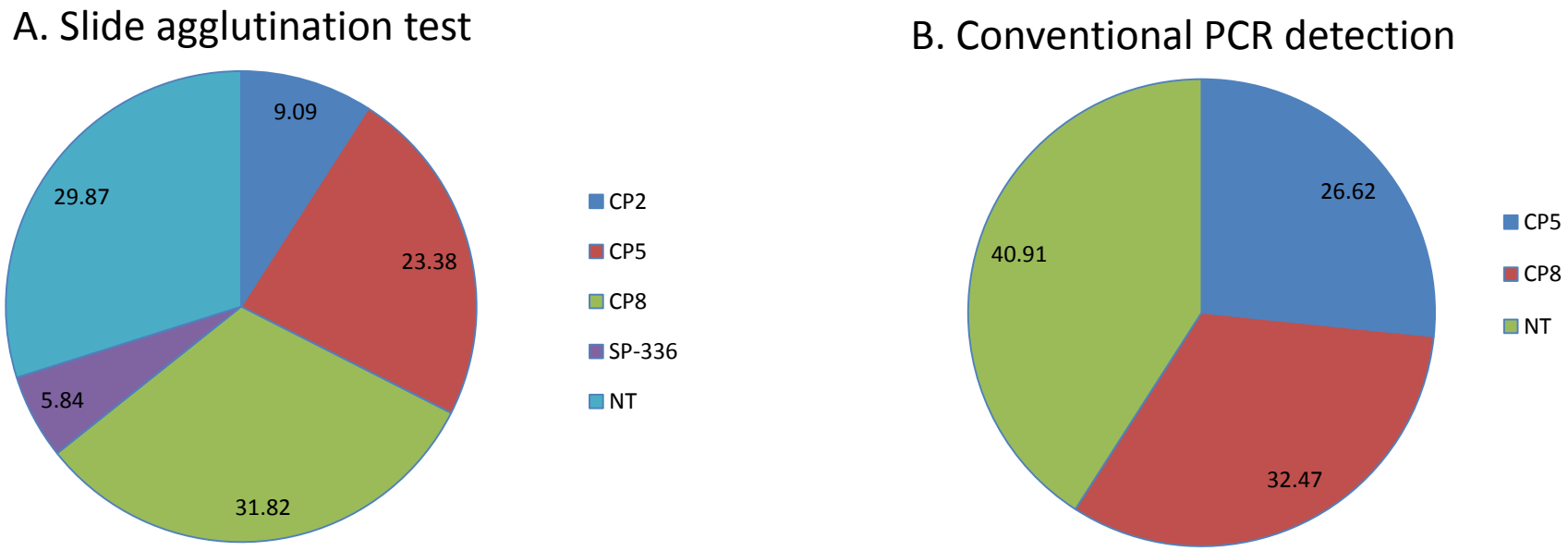
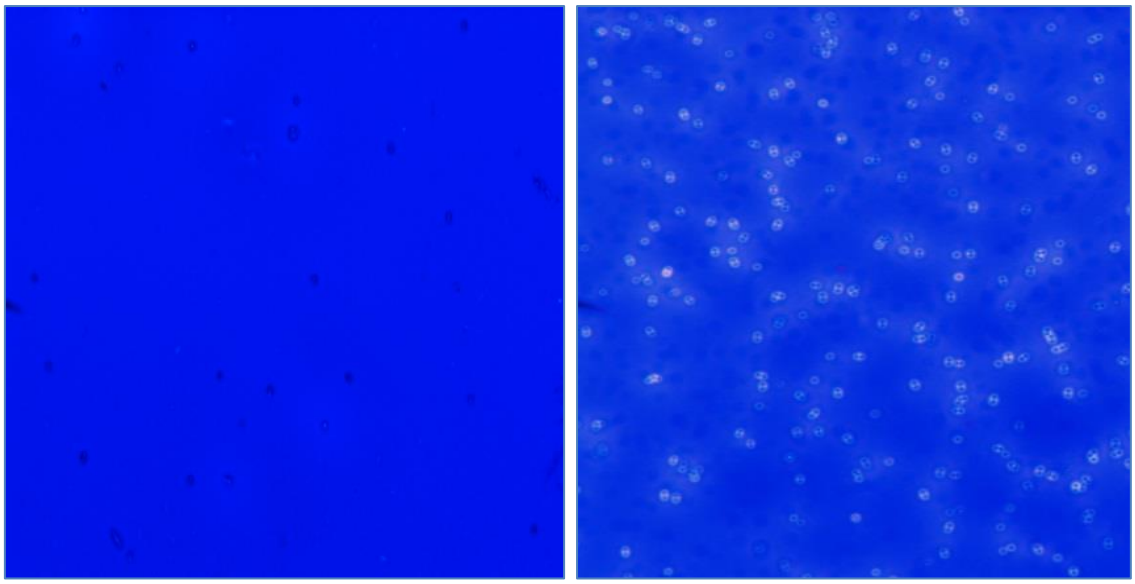


Figure 5.4: Amplification of *cap5* and *cap8* loci in PCR

Lane 1 – Fermentas DNA Ladder, O'Range Ruler 50bp
Lane 2 – *cap5*, 880bp (Moore and Lindsay et al., 2001)
Lane 3 – *cap8*, 173bp (Verdier et al., 2007)
Lane 4/5 – Negative controls
Lane 6 – Fermentas DNA Ladder, O'Range Ruler 50bp

Figure 5.5: Demonstration of encapsulated and non-capsulated *S. aureus* by negative staining



A. Non-capsulated *S. aureus* B. Capsulated *S. aureus*

Chapter 6: Standardisation of a non-invasive mouse mastitis model by using different phenotypes of *Staphylococcus aureus*

6.1 Introduction

Mastitis is one of the major infectious diseases of dairy cows causing huge economic loss to the dairy industry worldwide (Halasa et al., 2007). Worldwide implementation of mastitis control programme though initially reduced the incidence of mastitis, it still remains an uncontrollable contagious disease worldwide mainly due to failure of mastitis control (Bradley, 2002), development of antibiotic resistance by some pathogens (Hillerton and Berry, 2005), increase international pressure to limit the use antibiotics due to its associated hazard to human health (Notebaert and Meyer, 2006). The pathogenesis of mastitis has been extensively studied in the last few decades with ultimate aim of prevention and control of bovine mastitis. However, more research is needed on mechanisms of damage to the mammary glands and mechanisms of immunity against infection of the mammary gland to study the mode of action of mastitis-associated pathogens, immune mechanism in host and efficacy of vaccine trials.

The use of large animal models including cow, goat and sheep, to study bovine mastitis has its associated problems such as cost and management, even using minimal number of animals. Besides, only limited number of hypotheses can be studied in large animals (Brouillette and Malouin, 2005). An alternative laboratory animal model which is cost effective and can easily be maintained in laboratory would contribute immensely to mastitis research. Experimental mouse mastitis model was first described and characterised by Chandler in 1969 (Chandler, 1969, 1970). Mouse is still considered a suitable animal model for bovine mastitis research due to cost effectiveness, minimum management and similarity between mouse and cow's mammary glands in respect to neutrophil infiltration and tissue damage (Brouillette and Malouin, 2005). Both the species have two pairs of anatomically and functionally independent mammary glands in inguinal region (Notebaert and Meyer, 2006). In addition to these two pairs, mouse has three additional pairs of mammary gland in the thoracic region which can be used to study additional parameters in mastitis research (Notebaert and Meyer, 2006). Availability of large number of

laboratory animals at affordable cost is necessary for studying various parameters and prospective of mastitis, which also helps to secure valid statistics. Use of mouse mastitis model is useful when limited quantities of promising agents including vaccines, antimicrobials and immunomodulators are to be tested for their efficacy against bovine mastitis (Notebaert and Meyer, 2006). This model is particularly useful because of the potential of *S. aureus* to form biofilm on medical devices such as indwelling catheters and prostheses (Fitzpatrick et al., 2005). Biofilm formation by *S. aureus* in the bovine mammary gland may also assist in the establishment of bovine mastitis by promoting colonisation, persistence of the pathogen (Heilmann et al., 1996) and inducing antibiotic resistance (Babra et al., 2013). The properties of resistance to antibiotics and evasion of phagocytosis by biofilms may contribute to the occurrence of chronic mammary gland infection (Cucarella et al., 2004).

Bovine mastitis is an inflammatory disease of mammary gland (Bannerman, 2009) in which bacterial invasion leads to the migration of neutrophils and possibly macrophages to the site of inflammation (Persson et al., 1993). *S. aureus* strains carrying superantigens including enterotoxins and toxic shock syndrome toxin are important virulent factors in *S. aureus* pathogenicity. These toxins are responsible to activate T-lymphocytes and mononuclear cells leading to production of cytokines (Ferens et al., 1998).The important inflammatory mediators in lactating cow's udder are Interleukin (IL)-1, IL-2, IL-4, IL-10, IL-12, interferon gamma (IFN-γ) and Tumour Necrosis Factor (TNF)-α. IL-1 and TNF-α help in accumulation of neutrophils at the site of infection. Many investigators have reported higher concentrations of IL-1 (Riollet et al., 2000; Bannerman et al., 2004) and TNF- α (Kauf et al., 2007) in milk during mastitis as part of the mediation of local and systemic inflammatory responses. The inflammatory cytokine, TNF-α, was not detected in quadrant assessment of the udders in healthy cows, however, high concentrations were detected both in blood and milk from cows suffering from bovine mastitis (Bannerman, 2009). TNF-α has been reported to facilitate endothelial activation leading to migration of neutrophils to the site of infection (Brouckaert and Fiers, 1996).

Various intra-mammary inoculation techniques have been used to produce mastitis involving experimental animals. One technique involves inoculation of bacterial suspension by dropping it on the tip of mammary teat and allowing absorption (Anderson, 1979). Another technique inoculates bacterial pathogen by passing a micropipette of less than 75μm diameter through teat canal (Lee et al., 2003). A commonly used method is the injection of inoculum by using a 30 gauge needle after incising the near end of teat ((Brouillette and Malouin, 2005). The fourth technique of inoculation involves surgical exposure of mammary gland followed by injection of bacterial suspension into primary duct by using a 30 gauge needle (Nguyen et al., 2000). However, this method is not suitable for lactating and pregnant mice as streak canal can hardly be located due to mammary tissue proliferation.

The objective of this research was to (a) investigate the association of biofilm formation of *S. aureus* in the pathogenesis of bovine mastitis using the mouse mastitis model (b) determine level of cytokines primarily of IL-1β, IL-6 and TNF-α, using serum samples of infected mice, other cytokines including IL-10, IL-12, IL-17A and IFN-γ were also measured (c) standardise a mammary gland inoculation technique in which, the mammary glands (L5 and R5) are inoculated without traumatising the teat with the intention of mimicking the natural mode of infection, and (d) semi-quantitatively measure the histological changes in host animals to estimate the level of infection and inflammatory response. CP8-positive *S. aureus* strains were selected for the experiment due to their predominance in both clinical and subclinical cases of bovine mastitis in Australia (Gogoi-Tiwari et al., 2014).

6.2. Materials and methods

6.2.1 Bovine *S. aureus* phenotypes

A total of four encapsulated (CP8) *S. aureus* strains, isolated from mastitic milk of cows, with strong or weak biofilm forming potential, were used in this investigation (Table 6.1). These strains were selected from a collection of 154 strains, obtained from Victoria and Queensland, Australia.

Table 6.1: Properties of *S. aureus* strains used in the study

Group	*S aureus* strain	Capsular polysaccharide type	Biofilm formation (TCP/CRA method)	Presence of *ica* genes *icaA* *icaD*	Detection of genes encoding enterotoxins A to J genes or production	Presence of Alpha (*hla*) and *beta* (*hlb*) toxin genes	Exfoliative toxins ETA ETB	PVL	TSST-1
1	BOAISRF *S. aureus* 51	CP8	Strong biofilm	+ve +ve	SEH (*seh*)	*hla, hlb*	-ve -ve	-ve	-ve
2	BOAISRF *S. aureus* 117	CP8	Strong Biofilm	+ve +ve	SEG (*seg*), SHE (*she*), SEI (*sei*)	*hla, hlb*	-ve -ve	-ve	-ve
3	BOAISRF *S aureus* 48	CP8	Strong biofilm	+ve +ve	None	*hla, hlb*	-ve -ve	-ve	-ve
4	BOAISRF *S. aureus* 104	CP8	Weak biofilm	-ve -ve	None	*hla, hlb*	-ve -ve	-ve	-ve
7	Control	Normal saline	-	-	-	-	-	-	-

6.2.2 Capsular typing of *S. aureus* isolates

CP typing of the *S. aureus* strains isolated from bovine mastitis was carried out using molecular as well as serological methods as already described in Chapter 5, Section 5.2.4 and 5.2.5 of this thesis.

6.2.3 Determination of biofilm forming potential of *S. aureus* isolates

6.2.3.1 Biofilm assay

Determination of biofilm forming characteristics of the strains were determined using the Congo red agar (CRA) method (Freeman et al., 1989) and the Tissue Culture plate as described in Chapter 3, Section 3.2.3 of this thesis.

6.2.4 *ica* typing of *S. aureus* isolates

ica typing of the two *S. aureus* isolates was performed as per the protocol described in chapter 3.

6.2.5 Determination of toxin production potential of *S. aureus* isolates

Determination of different toxin forming potential of the strains by using PCR was discussed in Chapter 4, Section 4.25 of this thesis.

6.2.6 Infection of mammary gland

6.2.6.1 Preparation of bacterial inocula

The *S. aureus* phenotypes were grown on MH agar plates at 37°C for 18h. The organisms were washed from the plates using 20 ml of isotonic saline and suspended in isotonic saline to give a final viable bacterial count of 4×10^{11} ml^{-1} (Anderson and Chandler, 1975).

6.2.6.2 Animal ethics approval

All animal work described in this investigation was approved by the Animal Ethics Committee of Curtin University (Approval number: AEC_2011_65) prior to commencement of the experiment. The mice were used for the study ensuring compliance with the Western Australian Animal Welfare Act 2002.

6.2.6.3 Animals

A total of 20 Balb/c first-pregnancy mice, in five groups of 4 mice each were used

for the experiment. Pups were removed from the lactating mice when they were between 5-15 days old, approximately 1h prior to the experiment. The pups were euthanized and not allowed to suckle after inoculation of the mammary gland with the *S. aureus* strains or normal saline in case of the control group of mice.

6.2.6.4 Method of infection of the mammary gland

Inoculation of the different *S. aureus* phenotypes into mammary glands was carried out using a modification to the procedure described previously (Brouillette et al., 2004) by eliminating the incision of the proximal end of the teat was made to expose the teat canal, and inoculating the *S. aureus* strains directly into the mammary duct. Briefly, mice were anaesthetised using 100 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine administered by the intraperitoneal route and laid in the supine position. The area surrounding the 5^{th} pair of mammary glands (L5 and R5) was disinfected with 70% ethanol. A binocular dissecting microscope was used to locate the duct orifice of the teat. While controlling the teats with sterile forceps, 0.05 ml of bacterial suspension equivalent to 2x 10^{10} CFU *S. aureus* was injected using a blunt smooth 31-gauge hypodermic needle to a depth of not more than 4mm. The infection was allowed to progress for 48 h and the mice were observed at six-hour intervals to assess development of macroscopic clinical signs of infection. All animals were administered one dose of Buprenorphine hydrochloride (0.05-0.1 mg kg^{-1}) subcutaneously pre-operation so that anticipated pain relief was available for up to 12 h post bacterial inoculation. A control group of mice was injected with normal saline using the same procedure.

6.2.7 Post inoculation examination

6.2.7.1 Macroscopic examination

The mammary glands of mice were observed for clinical signs of mastitis including redness, swelling and discolouration of mammary gland and extrusion of exudate with or without squeezing of the mammary gland. Monitoring of mice for morbidity and mortality were carried out at 6 hourly intervals up to 48h. The level of clinical signs was graded as 0 (no macroscopic changes), + (low) grade, ++ (medium grade) and +++ (severe grade) based on the observed clinical features including redness, swelling, and discolouration of mammary gland, exudate, morbidity and mortality.

Mice were euthanized 48h after inoculation, and investigated for level of infection by determining the bacterial load (Anderson and Chandler, 1975) as described below and the mammary gland tissue collected for estimating the level of inflammation and histological changes (Anderson and Chandler, 1975).

6.2.7.2 Bacteriological procedure

6.2.7.2.1 Mammary gland

At post-mortem examination, the L5 mammary glands from control and test mice were collected and individually ground in sterile Griffith's tubes containing 2 ml of sterile normal saline. The homogenates from the mammary glands were subjected to serial tenfold dilutions and inoculated on Baird Parker (BP) agar plates (Pathwest, Laboratory Medicine, WA) by the spread plate method and incubated at 37°C for 48h, followed by determination of colony counts of *S. aureus* per mammary gland (Fig 6.2).

6.2.7.2.2 Blood, liver, lung and spleen

Blood samples obtained by cardiac puncture and organs including liver, lung and spleen were inoculated on BP agar plates by streaking for isolated colonies and incubated at 37°C for 48 h.

6.2.7.3 Histological procedure

6.2.7.3.1 Mammary gland

After aseptically shaving the hair around the gland the R5 mammary glands were collected for histological examination. The R5 glands were fixed in 10% neutral buffered formalin for 24 h, processed on an automatic tissue processor and embedded in paraffin wax. Sections were cut at 4 µm thickness at three levels and stained by the Haematoxylin and Eosin stain (Bancroft and Gamble, 2008). An additional section was stained for bacteria using Gram Twort Method (Twort, 1924; Ollet, 1947).

6.2.7.3.2 Blood

Blood smears were prepared following standard procedure and stained by the Diff Quik method (Skipper and Destephano, 1989).

6.2.8 Grading of histological changes observed in mammary glands

The histopathological changes observed in mammary glands of mice, injected with different strains of *S. aureus*, were graded as follows:

Level 0: No reaction (Fig 6.1a).

Level 1: Organisms identified with minimal inflammatory response in mammary tissue (Fig 6.1b).

Level 2: Moderate inflammation in peri mammary and intra mammary tissue with intra luminal organisms observed (Fig 6.1c).

Level 3: Marked inflammatory cell infiltration into mammary tissue in the presence of organisms with evidence of tissue degeneration including necrosis (Fig 6.1d).

6.2.9 Quantification of inflammatory cytokines

6.2.9.1 Procedure

Quantification of inflammatory cytokines, IL-1β, IL-10, IL-12, IL-17A, IFN-γ and TNF-α was performed by using BD cytometric Bead Array (CBA) Mouse/Rat soluble protein Master Buffer Kit (BD Biosciences), USA. Preparation of Mouse/ Rat soluble protein flex set standards, capture beads and detection reagents were performed as per the standard protocol provided with the reagent kit. Briefly, 50µL of Mouse/Rat soluble protein flex set standard dilutions ranging from 1:2 to 1:256 and one negative control containing only assay diluent was prepared. To 10µL of each unknown serum sample, 10µL of each capture bead and mixed PE detection reagent was added. Tubes were incubated at 4°C for one hour each after addition of capture beads and PE detection reagent protected from light. Following incubation, 200µL of wash buffer was added to each tube and centrifuged at 200g for 5min. The supernatant was aspirated carefully and discarded. The remaining pellet was reconstituted using 200µL of wash buffer and analysed using Attune Acoustic Focussing Flow Cytometer using the FlowJo software.

6.2.10 Statistical analysis

Statistical analysis was carried out using STATA v11 (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP) for comparing total viable counts of *S. aureus* recovered from mammary glands 48 h post-harvest

and the grade of histopathological changes with the biofilm formation of the *S. aureus* strains.

To compare the TNF-α levels between groups of mice injected with strong biofilm and weak biofilm forming *S. aureus* phenotypes student's t-test was performed. Statistical significance was set at $p < 0.05$.

6.3 Results

6.3.1 Macroscopic examination of mammary glands

The control group showed no macroscopic changes in the inoculated glands. All of the test mice showed varying degrees of change in the gross appearance of mammary glands (Table 6.2).

Table 6.2: Clinical signs observed in different groups of inoculated mice (Observations up to 48 hours post inoculum)

Level of clinical signs* observed in test mice injected with different *S. aureus* phenotypes**				
Time post inoculation	***S. aureus* 51**	***S. aureus*117**	***S. aureus* 48**	***S. aureus* 104**
6h	0	0	0	0
12h	+	+	+	+
18h	++	++	++	+
24h	++	++	++	+
30h	+++	+++	+++	++
36h	+++	+++	+++	++
42h	+++	+++	+++	++
48h	+++	+++	+++	++

*Clinical features include redness, swelling, and discolouration of mammary gland, exudate, morbidity and mortality. Grade scores compare observed features to the most severe changes: 0 - no macroscopic changes, + low grade, ++ medium grade, +++ severe grade.

** Column representing mice injected with normal saline by the intramammary route is not presented because of the absence of histopathological changes.

6.3.2 Bacterial load and histopathological changes of mammary gland

The bacterial loads in the mammary glands as a measure of proliferation of *S. aureus* at the injection site and the associated histopathological grade observed in the mammary tissue are shown in Table 6.3 and Figures 6.1 and 6.2. The bacteriological load study demonstrated that mice injected with CP8 *S. aureus* strains with strong biofilm forming ability (*S. aureus* 51, 117 and 48) had higher bacterial count than the CP8 weak biofilm producing strain (*S. aureus* 104). A one-way ANOVA between the mice groups injected with the four *S. aureus* strains showed statistically significant differences in bacterial load between the groups ($P < 0.001$). A Bartlett's test for equal variances showed that the variances of the four groups were not unequal ($P > 0.05$). A pairwise comparison analysis between each group adjusted for multiple-comparison using the Bonferroni correction showed that the mice group injected with the *S. aureus* 51 strains had the highest load ($P < 0.001$). Histopathological studies showed that mice injected with CP8 *S. aureus* with strong biofilm forming characteristics (*S. aureus* 51, 117 and 48) produced severe mastitis lesions characterised by level 3 histopathological lesions, showing a profuse inflammatory infiltrate with severe tissue damage including necrosis in mammary tissue. On the other hand, *S. aureus* strain 104 with weak biofilm forming ability demonstrated only level 2 histopathological changes (Table 6.3).

6.3.3 Bacteriology of blood and and histopathology of liver, lung and spleen

The culture of blood and organs (liver, lung and spleen) in BP agar plates was negative for *S. aureus* consistent with no evidence of systemic infection. No overt microscopic evidence of inflammation was observed in tissue sections of lung, liver and spleen from any of the mice.

6.3.4 Quantification of inflammatory cytokines

Quantification data of inflammatory cytokines, IL-1β, IL-6, IL-10, IL-12, IL-17A, IFN-γ and TNF-α is presented in Table 6.4. The result showed that the levels of TNF-α were significantly higher ($p<0.05$) in the sera of mice inoculated with CP8-positive, strong biofilm forming *S. aureus* than those inoculated with weak biofilm forming CP8-positive *S. aureus*.

6.4 Discussion

Various investigators have reported the importance of CP (Thakker et al., 1998; Tuchscherr et al., 2005) and biofilm (McKenney et al., 1999, Rupp et al., 1999) as virulence factors in the pathogenesis of *S. aureus* in different animal models including the mouse model. This study aimed to assess the importance of biofilm as virulence factor of *S. aureus* in bovine mastitis using a mouse mastitis model. Given the production greater mammary tissue damage (level/grade 3 histopathological changes by the strong biofilm-producing *S. aureus* strains versus level/grade 2 lesions by the weak biofilm-producing strain, also verified by the lack of detection of the *ica*A and *ica*D), it is clear that strength of the biofilms produced by *S. aureus* appeared to contribute to the mammary tissue damage. This observation suggests the inclusion of antigens contributing significantly to biofilm formation in a cocktail vaccine formulation for prevention of bovine mastitis.

The bacteriological load study demonstrated higher bacterial count in the mice injected with CP8 strong biofilm forming *S. aureus* than the mice injected with CP8 weak biofilm former. However, the bacterial load values were significantly different from one another (Table 6.3); suggest a lack of correlation between *S. aureus* load in the mammary gland and associated tissue damage. However, it appears that mammary tissue damage may be associated with the biofilm forming ability of *S. aureus* as there was significantly different bacterial load between the biofilm former and non-biofilm former *S. aureus* injected mammary glands of mice.

Elevated levels of TNF-α concentration both in serum and milk of cows suffering from natural infection of *E. coli* mastitis has been reported (Nakajima et al., 1997; Hisaeda et al., 2001) suggesting a potential role for this cytokine in development of inflammation in mammary gland. The level of TNF-α in serum is highly elevated in acute cases of clinical mastitis (Nakajima et al., 1997) that has been observed to result in activation and migration of neutrophils (Blum et al., 2000). A sharp increase in mRNA transcription of TNF-α at 24 h followed by a decrease at 32 h post infection with *S. aureus* (Alluwaimi et al., 2003) has been reported. In our study, the level of TNF-α was significantly higher ($p<0.05$) in mice inoculated with the strong biofilm forming *S. aureus* than the weak biofilm forming strain even 48 h post

infection. This finding suggests the possible role of TNF-α in the damage caused by *S. aureus* to the mammary glands positively supporting the need for further studies on the role of inflammatory cytokines in acute and chronic bovine mastitis.

However, there was either no statistically significant difference between the levels of other cytokines except IL-10 and IL-12 produced by only one of the 3 strong biofilm forming strains (*S. aureus* 48) and the weak biofilm forming strain (*S. aureus* 104). Interestingly however the weak biofilm for ing strain (*S. aureus* strain 104) produces significantly more ($p<0.05$) IL-10 and IL-12 than the 2 other strong biofilm forming strains (*S. aureus* strains 51 and 117). Because of the inconsistency of this data, repeat studies are clearly warranted prior to hypothesizing their potential roles in the induction of mouse mastitis.

This study has demonstrated that mastitis can be reliably produced in a mouse model by direct inoculation of *S. aureus* into the mammary gland via the mammary duct without traumatising the mammary gland surface epithelium or glands. This approach was applied to mimic the most likely natural route of infection. The bacteriological and histopathological study revealed proliferation of the bacteria as well as inflammatory infiltrates in mammary glands. Use of a direct delivery inoculum in a mouse mastitis model may minimise the chance of secondary bacterial infection, which can occur in the case of invasive mouse mastitis model where the tip of the mammary gland is incised (Brouillette and Malouin, 2005; Brouillette et al., 2005). The secondary bacterial infection may be caused by different microorganisms including *S. aureus* and those in the environment, cage litter or from the hands of animal handlers while picking up the animals to observe the mammary glands for clinical symptoms. Establishment of this mouse mastitis model provides an opportunity to determine the identity of *S. aureus* antigens responsible for induction of inflammatory cytokines with a view to formulation of a cocktail vaccine capable of reducing the levels of the inflammatory cytokines and potentially preventing or significantly reducing damage to the mammary gland.

Table 6.3: Total viable counts of *S. aureus* recovered from mammary glands 48 h post-harvest and the grade of histopathological changes

Group	S. aureus phenotype	Total number of mammary glands investigated	Log average number of bacteria (CFU) recovered from mammary glands ± SE	Grades for histopathological changes			
				M*1	M2	M3	M4
1	*S. aureus* 51	4	8.08 ± 0.002	3	3	3	3
2	*S. aureus* 117	4	7.72 ± 0.003	3	3	3	3
3	*S. aureus* 48	4	7.92 ± 0.001	3	3	IF**	2
4	*S. aureus* 104	4	7.52 ± 0.009	2	2	2	2
5	Control (NS***)	4	0	0	0	0	0

M*= Mammary gland, IF**= Inoculation failed, NS***= Normal saline

In one-way ANOVA between the mice groups ($P < 0.001$), Bartlett's test for equal variances for four groups was unequal ($P > 0.05$), pairwise comparison analysis between each group adjusted for multiple-comparison using the Bonferroni correction showed that the mice group injected with the *S. aureus* 51 strains had the highest load ($P < 0.001$).

Table 6.4: Detection of levels of various inflammatory markers in sera samples of mice 48 h post-infection

Group	*S. aureus* phenotype	IL-1β Pg/mL± SE	IL-6 Pg/mL± SE	IL-10 Pg/mL± SE	IL-12 Pg/mL± SE	IL-17A Pg/mL± SE	IFN-γ Pg/mL± SE	TNF-α Pg/mL± SE	Histopathology grade
1	*S. aureus* 51	3.6405±0.35	1.9±1.1	5.50±0.89	2.18±0.57	0.10±0.02	9.85±1.19	13.0045±0.50	3
2	*S. aureus* 117	3.62025±0.25	5.1±1.9	4.36±0.28	1.43±0.35	1.16±0.02	7.76±0.27	20.6285±4.57	3
3	*S. aureus* 48	4.04175±0.79	16.9±14	12.70±0.72	12.24±1.25	2.64±0.13	7.08±0.1	33.874±14.3	2.7
4	*S. aureus* 104	3.19175±0.59	33.4±9	7.28±0.84	8.75±0.76	1.67±0.26	11.03±1.67	8.85475±2.04	2
5	Control (NS)	0	0	0	0	0	0	0	0

In student's t-test for TNF-α levels between groups of mice injected with strong biofilm (*S. aureus* 51, 117, 48) and weak biofilm forming *S. aureus* phenotypes (*S. aureus* 104) showed high TNF-α ($p < 0.05$) level in mice injected with strong biofilm forming *S. aureus.*

Figure 6.1: Histopathological changes observed in mammary tissue

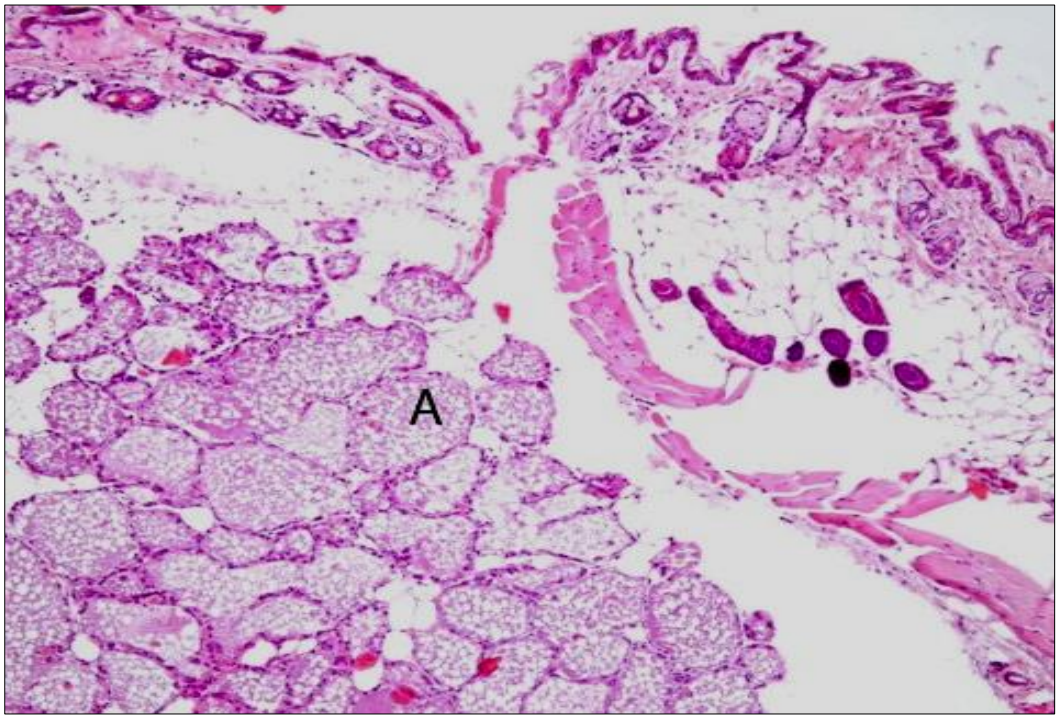


Figure 6.1(a): Mouse mammary tissue. Control animal. No evidence of inflammatory response in mammary tissue (A) to sterile injection. H&E x100mag

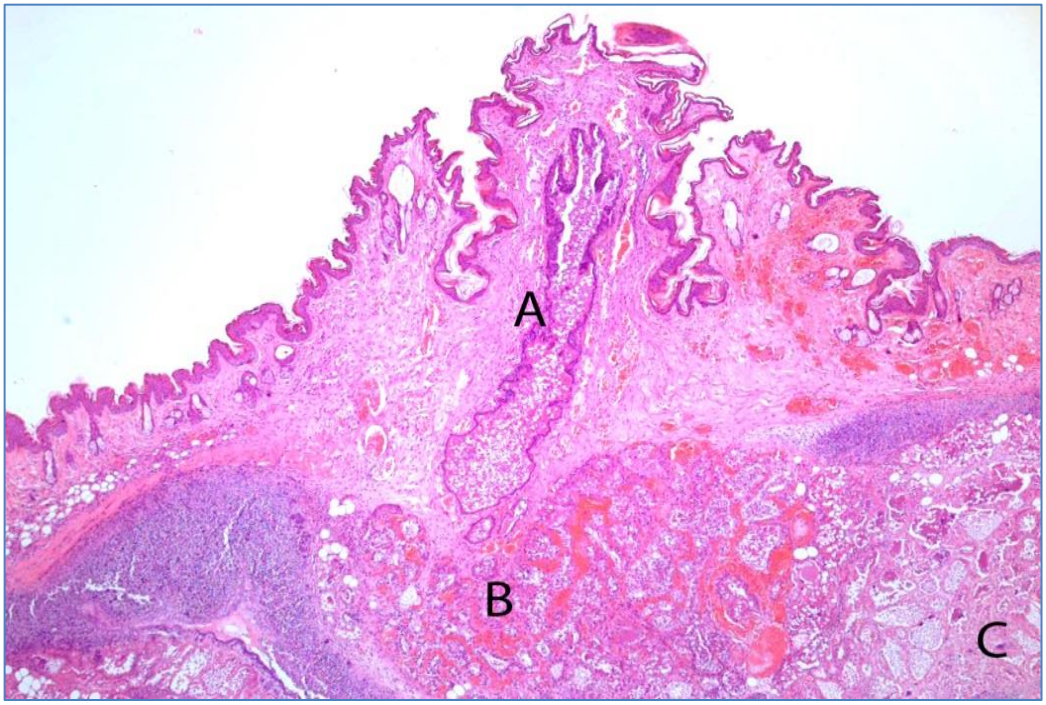


Figure 6.1(b): Level 1 inflammation. Mouse teat and mammary tissue. Inflammatory response induced by injection of *S.aureus* via the mammary duct. Note exudate in duct (A) , acute response in mammaryglands (B) including necrosis (C). H&E x40mag

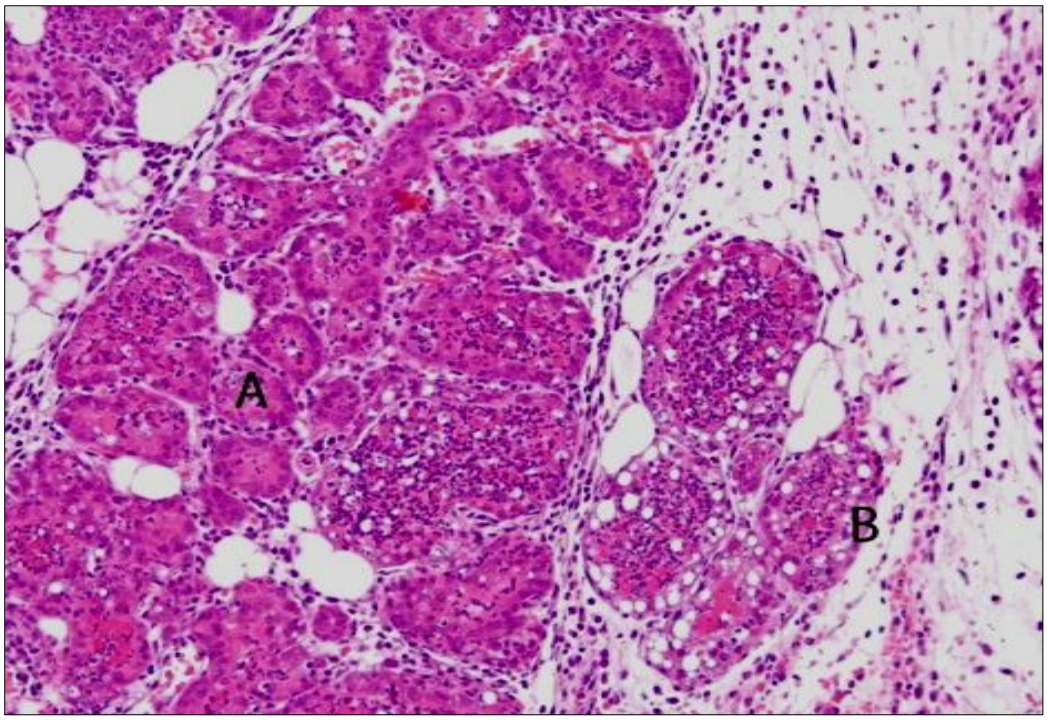


Figure 6.1(c): Mouse mammary tissue. Level 2 inflammation.Mammary glands (A)Infiltration of acute inflammatory cells in supporting connective tissue and intraluminal space (B). H&E x200mag

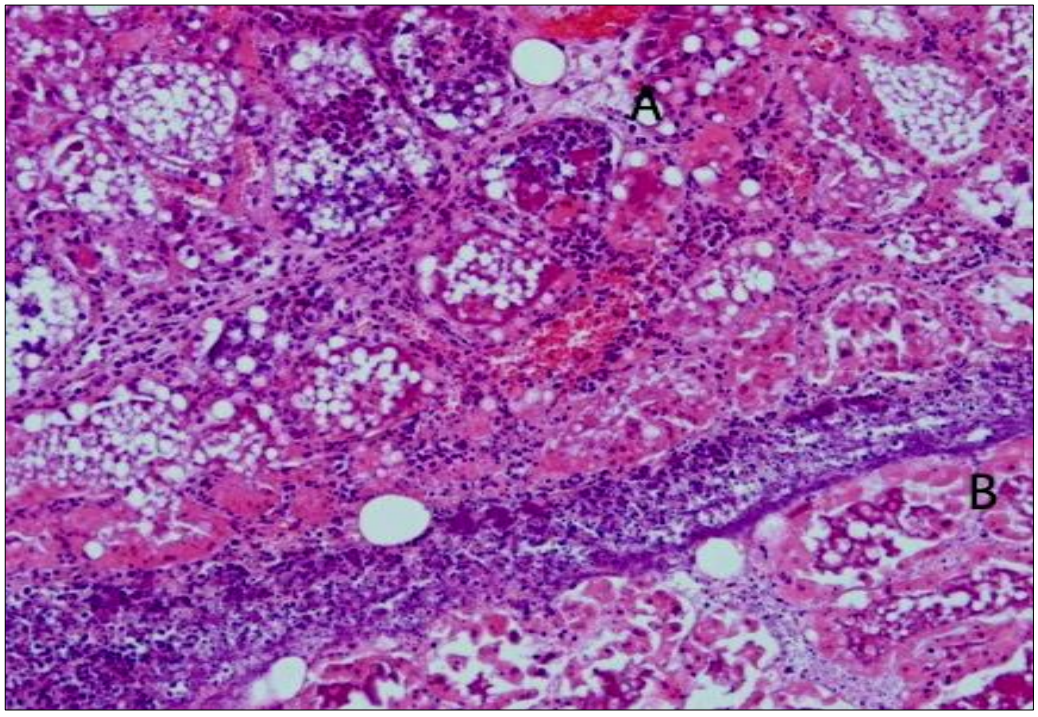


Figure 6.1(d): Mouse mammary tissue. Level 3 inflammation. Marked acute inflammatory cell infiltration (A) with tissue necrosis (B). H&E x200mag,

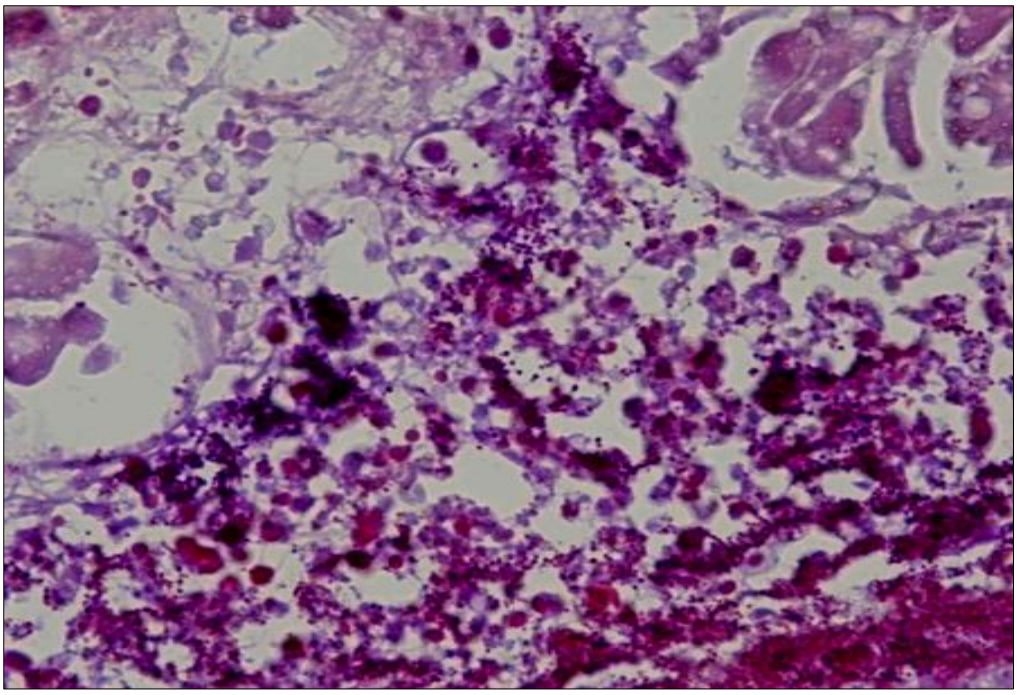


Figure 6.1(e): Mouse mammary tissue. Infiltrate detail. Gram positive bacteria and associated inflammatory cell exudate. Gram Twort x400mag.

Figure 6.2: Standard plate count of *S. aureus* colonies in Baird Parker agar medium

Chapter 7: Mammary tissue damage caused by a non-typeable *Staphylococcus aureus* strain using the mouse mastitis model and assessment of the potential of anti-inflammatory cytokine or cytokine receptor antibodies in the prevention of mortality

7.1 Introduction

The predominant capsular types in *S. aureus* from human source are reported to be CP5 and CP8, with non typeable strains representing less than 25% (Lee and Lee, 2000). In contrast, *S. aureus* isolates from bovine mastitis cases have been either encapsulated are 14-40% of isolates (Sompolinsky et al., 1985; Poutrel et al., 1988; Han et al., 2000; Sordelli et al., 2000). While investigating the prevalence of *S. aureus* CP types associated with bovine mastitis cases in Australia, 30% of the isolates were detected serologically to be nontypeable (Gogoi-Tiwari et al., 2014). Essentially no attempts have been made to develop vaccines against non-typeable (NT) isolates. However, given that NT strains can persist in the mammary gland for longer duration than the encapsulated strains and can invade mammary epithelial cells in higher numbers than the encapsulated ones (Tuchscherr et al., 2005), the only alternative left is treatment with antibiotics.

While standardizing the non-invasive mouse mastitis model for bovine mastitis, it was discovered that damage caused to the mammary gland by infection with test encapsulated *S. aureus* was associated with the strength of biofilm formation with an increase in TNF-α level (Refer Chapter 6). It was therefore necessary to determine if the same held true for the NT *S. aureus* strains and whether administration of antibodies against the inflammatory cytokine produced and its receptor could prevent mortality in mice infected with strong biofilm forming strain.

The aims of this experiment, therefore, were:

(a) To determine the virulence of non-typeable strain with strong versus weak biofilm forming ability as measured by the level of tissue damage in the mammary glands of infected mice using a mouse mastitis model.

(b) To determine the production of various inflammatory biomarkers in sera samples of infected mice

(c) To treat mice with antibodies against the predominant cytokine produced and its receptor to reduce mammary tissue damage and prevent mortality, if any.

7.2 Material and Methods

7.2.1 Bovine *S. aureus* phenotypes

A total of two non-typeable *S. aureus* strains, isolated from mastitic milk of cows, with strong or weak biofilm forming potential, were used in this investigation (Table 7.1).

7.2.2 Capsular typing of *S. aureus* isolates

Capsular typing of the two *S. aureus* strains used in this investigation was carried out using molecular as well as serological methods as described in Chapter 5 Section 5.2.4 & 5.2.5.

7.2.3 Determination of biofilm forming potential of *S. aureus* isolates

Biofilm forming potential of the two nontypeable strains were determined by Congo red agar (CRA) method (Freeman et al., 1989) and the Tissue Culture plate method as described in Chapter 3 Section 3.2.3.

7.2.4 *ica* typing of *S. aureus* isolates

ica typing of the two *S. aureus* isolates was performed as per the protocol described in Chapter 3 Section 3.2.4.2.

7.2.5 Determination of toxin production potential of *S. aureus* isolates

Determination of different toxin forming potential of the strains by using PCR was discussed in Chapter 4, Section 4.2.5 of this thesis.

7.2.6 Infection of mammary gland using non typeable *S. aureus* strains

7.2.6.1 Preparation of bacterial inocula

The *S. aureus* phenotypes were grown on MH agar plates at 37°C for 18h. The organisms were washed from the plates using 20 ml of isotonic saline and suspended in isotonic saline to give a final viable bacterial count of 4×10^{11} ml^{-1} (Anderson and Chandler, 1975).

Table 7.1: Phenotypes of *S. aureus* strains used in the study

Group	*S aureus* strain	Capsular polysaccharide type	Biofilm formation (TCP/CRA method)	Presence of *ica* genes *icaA* *icaD*	Detection of genes encoding enterotoxins A to J genes or production	Presence of Alpha (*hla*) and *beta* (*hlb*) toxin genes	Exfoliative toxins ETA ETB	PVL	TSST-1
1	BOAISRF *S. aureus* 83	Non-capsulated	Strong biofilm	+ve +ve	SEC (*sec*), SED (*sed*), SEG (*seg*), SEI (*sei*)	*hla, hlb*	-ve -ve	-ve	-ve
2	BOAISRF *S. aureus* 87	Non-capsulated	Weak biofilm	-ve -ve	none	*hla, hlb*	-ve -ve	-ve	-ve
3	Control	Normal saline	-	-	-	-	-	-	-

Table 7.2: Experimental design for treatment with anti-inflammatory cytokines

Treatment group	Number of mice	Control group (PBS)	Total number of mice
Anti-IL-6 antibody	**6**	**6**	**12**
Anti-IL-6β receptor antibody	**6**	**6**	**12**

7.2.6.2 Animal ethics approval

All animal work described in this investigation was approved by the Animal Ethics Committee of Curtin University (Approval number: AEC_2011_65) prior to commencement of the experiment. The mice were used for the study ensuring compliance with the Western Australian Animal Welfare Act 2002.

7.2.6.3 Animals

A total of 12 Balb/c first-pregnancy mice, in three groups comprising 4 mice/group were used for the experiment. Pups were removed from the lactating mice when they were between 5-15 days old, approximately 1h prior to the experiment. The pups were euthanized and not allowed to suckle after inoculation of the mammary gland with the *S. aureus* strains or normal saline in case of the control group of mice.

7.2.6.4 Method of infection of the mammary gland

Inoculation of the different *S. aureus* phenotypes into mammary glands was carried out as per the method described in the Chapter 6, Section 6.2.4.4.

7.2.6.5 Post inoculation examination

All the postinoculation examinations including macroscopic examination, bacterial load study and histopathological study of mammary glands were carried out as per the methods described in Chapter 6, Section 6.2.5.1, 6.2.5.2.1, 6.2.5.3.1, respectively.

7.2.7 Quantification of inflammatory cytokines

Quantification of proinflammatory cytokines, IL-1β, IL-6, TNF-α, IFN-γ and other cytokines (IL-10, IL-12 and IL-17A) was performed by using BD cytometric Bead Array (CBA) Mouse/Rat soluble protein Master Buffer Kit (BD Biosciences), USA. The detail method is described in Chapter 6, Section 6.2.7.1.

7.2.8 Experimental design for treatment of mice with anti-inflammatory cytokine, IL-6 and IL-6 receptor antibodies

7.2.8.1 Animal ethics approval

All animal work described in this investigation was approved by the Animal Ethics Committee of Curtin University (Approval number: AEC_2014_01) prior to

commencement of the experiment. The mice were used for the study ensuring compliance with the Western Australian Animal Welfare Act 2002.

7.2.8.2 Animals

A total of 24 lactating (7 days) were used in the study. Two different treatment groups comprising 6 lactating mice were used as treatment groups. Two control groups also comprised 6 lactating mice in each (Table 7.2).

7.2.8.3 Treatment of animals with anti-inflammatory cytokine or anticytokine receptor antibody

The mice were injected with 400µl (85 µg) of IL-6 and IL-6β receptor antibodies by intraperitoneal routes on 1st day. A booster injection comprising the same dose was injected on the next day by the same route. Mice were challenged in both L5 and R5 mammary glands with 0.05 ml of bacterial suspension equivalent to 2x 10^{10} CFU *S. aureus* was injected using a blunt smooth 31-gauge hypodermic needle to a depth of not more than 4mm. The detail of the method is described in Chapter 6, Section 6.2.4.4. The mice were observed at one-hour intervals to assess development of macroscopic clinical signs of infection and mortality.

7.2.9 Statistical analysis

Statistical analysis was carried out using student's *t*-test to compare total viable counts of *S. aureus* recovered from mammary glands injected with non typeable strong biofilm forming *S. aureus* and weak biofilm forming *S. aureus*. *t* test was also performed to compare the IL-1β, IL-6, IL-12, IL-17, TNF-α and IFN-γ levels between groups of mice injected with *S. aureus* phenotypes. Statistical significance was set at $p < 0.05$.

7.3 Results

7.3.1 Infection of mammary gland using non-typeable *S. aureus* strains

7.3.1.1 Observation of mammary gland for clinical symptoms

The control group of mice injected with PBS did not show any clinical symptoms and the mammary glands appeared normal. Both the test groups of mice vaccinated with *S. aureus* 83 and 87 strains showed medium grades of clinical symptoms.

However mice injected with *S. aureus* 83 died 24 h of post inoculation and the group injected with *S. aureus* 87 died 30 h post inoculation (Table 7.3).

Table 7.3: Clinical signs observed in different groups of mice post-infection (observations up to 30 h post inoculation)

Time post inoculation	*S. aureus* 83	*S. aureus* 87	PBS (Control)
6 h	0	0	0
12 h	+	+	0
18 h	++	+	0
24 h	++/death	++	0
30 h		++/death	0

*Clinical features include redness, swelling, and discolouration of mammary gland, exudate, morbidity and mortality. Grade scores compare observed features to the most severe changes: 0 no macroscopic changes, + low grade, ++ medium grade, +++ severe grade.

7.3.1.2 Bacterial load and histopathological changes of mammary gland

The log average number of bacteria (CFU) isolated from the mammary glands of all the three groups of mice including control group and the associated histopathological changes in the mammary glands are presented in Table 7.4. The histopathological changes noted in mammary tissue are shown in Figure 7.1.

Table 7.4: Total viable counts of *S. aureus* recovered from mammary glands after death of mice

Group	*S. aureus* phenotype	Total number of mammary glands investigated	Log average number of bacteria (CFU) recovered from mammary glands ± SE	Histopathology grade
1	*S. aureus* 83	4	8.23 ± 0.001	1
2	*S. aureus* 87	4	7.91 ± 0.003	1
3	Control (NS*)	4	0	0

NS*= Normal saline

7.3.1.3 Bacteriology of blood and histopathology of liver, lung and spleen

The culture of blood and organs (liver, lung and spleen) in BP agar plates was negative for *S. aureus* indicating no evidence of systemic infection. There was no evidence of inflammation in tissue sections of lung, liver and spleen from any of the mice.

7.3.1.4 Quantification of inflammatory cytokines in serum

Quantification study of inflammatory cytokines, IL-1β, IL-6, TNF-α and other cytokines including IL-10, IL-12, IL-17A and IFN-γ showed that the levels of IL-1β, IL-6, IL-12, IL-17 and IFN-γ were significantly higher ($p<0.05$) in the sera of mice inoculated with non-typeable strong biofilm forming *S. aureus* 83 than those inoculated with weak biofilm forming non typeable *S. aureus* 87 (Table 7.5).

7.3.2 Treatment of mice using anti-inflammatory cytokine IL-6 and anti cytokine receptor antibodies IL-6Rβ

The detailed results of studies of clinical signs, mortality, bacterial load and histopathological grades of mammary glands in anti-inflammatory cytokine treated mice are presented in Table 7.6. The histopathological changes observed in mammary tissue are presented in Figure 7.1-7.4.

7.4 Discussion

Studies carried out with CP8 positive strong versus weak biofilm forming *S. aureus* isolates revealed that the extent of mammary tissue damage was associated with the strength of biofilms formed by *S. aureus* strains (Refer Chapter 6). This study was undertaken to compare the pathogenicity of two non-typeable *S. aureus* strains with and without biofilm forming ability. The study also aimed to determine the potential of anti-inflammatory cytokine, IL-6 and IL-6 R β antibodies to prevent mortality in mice. Non-typeable strains of *S. aureus* can survive in the mammary gland for longer duration than the encapsulated strains (Tuchscherr et al., 2005). Higher degree of inflammation appeared to have been induced by encapsulated than the non typeable *S. aureus* strains, which leads to quick clearance of these cells by the host immune system. The non-typeable cells are quickly internalized by the mammary epithelial cells due to absence of capsule and thus they are protected from the action of

phagocytic cells (Tuchscherr et al., 2005). In the current study, both the strains produced identical clinical symptoms and mammary tissue damage. Both the strains produced moderate level of clinical symptoms of mastitis and level 1 histopathological lesion in the mammary gland. However, the bacterial load in the mammary glands of mice injected with the non typeable *S. aureus* 83 strain with biofilm forming ability was significantly higher (≤ 0.05) than the non-biofilm forming *S. aureus* strain 87 strain. The period of observation in the study was 48 h. However, both the test groups of mice died before 48h, the mice injected with *S. aureus* 83 strain survived only 24 h followed by the mice injected by *S. aureus* 87. Mice in the latter died at 30h post inoculation.

The epithelial cells of mammary gland can produce cytokines (Bannerman, 2009). One of the effective alternative approaches to treat bovine mastitis caused by *S. aureus* can be the use of anticytokines as immunotherapeutic agents. Cytokines being the important soluble defence mechanism of mammary gland acts as mediators as well as regulators of inflammatory mediator cytokines by promoting recruitment of neutrophils to the site of infection. A variety of cytokines are reported to be present in healthy and infected mammary gland. They are interleukins (IL), colony stimulating factors (CSF), interferon gamma (IFN-γ) and tumour necrosis factor (TNF) [Alluwaimi, 2004]. The interleukins which are associated with bovine mammary gland infections are IL1, IL2, IL6 and IL8 (Alluwaimi, 2004). There are various reports of upregulation of level of IFN-γ (Bannerman et al., 2004), IL-1β (Riollet et al., 2001), IL-6 (Riollet et al., 2001), IL-12 (Lee et al., 2006) and TNF-α (Dernfalk et al., 2007) in milk and serum. Availability of a variety of recombinant cytokines and also due to their proven immune-modulatory properties (Bannerman, 2009), these cytokines have opened new avenues in the area of therapy and prophylaxis of bovine mastitis. Various researchers have reported the prevention and clearance of inf**e**ction from mammary gland and enhancement of efficacy of antibiotic treatment in bovine mastitis due to use of cytokine therapy (Sordillo and Babiuk, 1991; Erskine et al., 1998; Takahashi et al., 2005). It has been demonstrated *in vitro* that neutrophils exceeding $5x10^7$ cells/mL of milk produce IL-1β, IL-12, IFN-γ and TNF-α (Sohn et al., 2007).

In the present study, the analysis of sera samples collected from mice immediately before death showed higher levels of IL-1β, IL-6, IL-10, IL-17A, IFN-γ and TNF-α (Table 7.5). It has been speculated that quick internalization of non-typeable *S. aureus* cells by the mammary epithelial cells have prevented the clearance of *S. aureus* from the mammary gland which provided scope for production of cytokines of different types. The highly elevated of level of various cytokines can lead to cytokine storm, a fatal immune response which may result in sudden death (Osterholm, 2005). It has been demonstrated that patients died due to cytokine storm had higher levels of anti-inflammatory cytokine, IL-10 and pro inflammatory cytokines, IL-1β, IL-6 and TNF-α in serum samples (Horst, 2002). In the present study, between both the groups of test mice, the mice injected with *S. aureus* 83 produced significantly higher levels of IL-1β, IL-6, IL-12, IL-17 and IFN-γ ($p < 0.05$) than the mice injected with *S. aureus* 87. The level of IL-6 in sera samples of mice injected with *S. aureus* 83 was extremely high (15479.9±532 Pg/mL) which could have been responsible for the death of the mice within mere 24 h post inoculation. The only phenotypic difference between both the strains was the biofilm forming ability. Though not conclusively, but it can be concluded that biofilm forming ability of non-typeable strain may play role in the virulence of the *S. aureus* strain in bovine mastitis.

Based on results of the cytokine analysis, anti-IL-6 and IL-6R β antibodies were selected to use for the treatment of the mice in the second animal trial. We hypothesized that anti IL-6 and IL-6R β antibodies will down-regulate the proinflammatory cytokine IL-6 resulting delayed or absence of mortality in mice. An experiment studying the prophylactic effect of IL-2 and IFN-γ in healthy mammary gland showed promising results in cows (Alluwaimi, 2004). However, it failed to prevent *S. aureus*-associated intramammary infections. In the current study, though the mortality of the mice could not be prevented completely, but the duration of survival was increased by to 12 h 30 min and 13 h 15 min in mice treated with anti-IL-6 and anti-IL-6R β antibodies, respectively. Severity of mastitis was also reduced in terms of clinical symptoms and bacterial load from the mammary glands. The clinical symptoms level has been reduced from medium to low grade and there was significant difference in the load of *S. aureus* in the mice treated with anti-IL6 and IL-6R β antibodies. However, the delayed mortality provides window for treatment

of mastitis given the administration of a suitable antibiotic with the extended timeframe. Clearly further research is needed to check the status of mortality if anti-cytokines or anti-cytokine receptor antibodies are used in combination with antibiotics to treat bovine mastitis caused by *S. aureus*.

Table 7.5: Detection of levels of different cytokine biomarkers in sera samples of mice after death of mice

Group	*S. aureus* phenotype	IL-1β Pg/mL ±SE	IL-6 Pg/mL ±SE	IL-10 Pg/mL ±SE	IL-12 Pg/mL ±SE	IL-17A Pg/mL ±SE	IFN-γ Pg/mL ±SE	TNF-α Pg/mL ±SE
1	*S. aureus* 83	321.7±23	15479.9±532	66.8±0.96	3.0±0.42	28.6±1.79	59.5±1.78	163.3±4.5
2	*S. aureus* 87	27.7±41	529±109	12.86±0.69	1.43±0.35	18.20±0.5	12.70±1.50	174.9±21
3	Control (NS)	0	0	0	0	0	0	0

In student's t-test for IL-1β, IL-6, IL-12, IL-17 and IFN-γ levels between groups of mice injected with *S. aureus* 83 and *S. aureus* 87 showed high IL-1β, IL-6, IL-12, IL-17 and IFN-γ ($p < 0.05$) levels in mice injected with *S. aureus* 83.

Table 7.6: Studies of clinical signs, mortality, bacterial load and histopathological grades of mammary glands in anti-inflammatory cytokine treated mice

Treatment group	Total number of mammary glands investigated	Clinical symptoms grading	Mortality (hour)	Log average number of bacteria (CFU) recovered from mammary glands after death of mice	Histopathology grade
IL-6	6	++	36h 30 min	7.64±.03	1
IL-6R β	6	+	37 h 15 min	7.58 ±.04	1
PBS control (IL6)	6	+	24h	8.29±0.02	1
PBS control (IL-6 R β)	6	+	24h	8.24±0.05	1

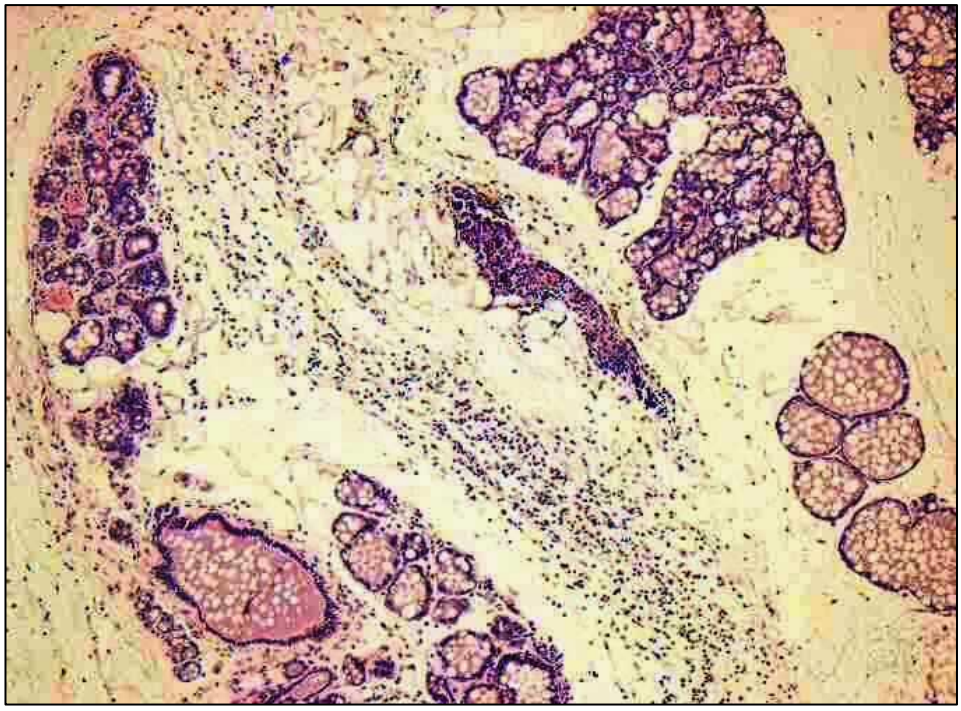


Figure 7.1: Mammary tissue specimen from lactating mouse injected with PBS and challenged using non-typeable *S. aureus* 83. Small numbers of interlobular inflammatory infiltrates in mammary tissue (A). Level 1 category inflammation. H&E x 100mag.

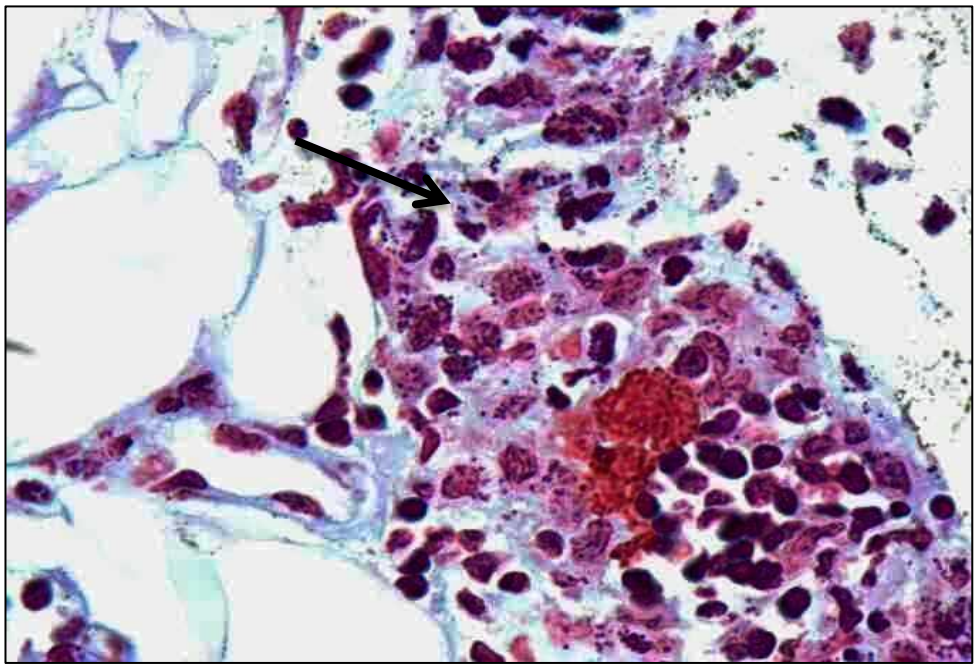


Figure 7.2: Mammary tissue specimen from lactating mouse injected with PBS and challenged using non-typeable *S. aureus* 83 (Figure 7.1). Gram positive bacteria (Arrow) within suppurative inflammatory material. Gram Twort x1000mag.

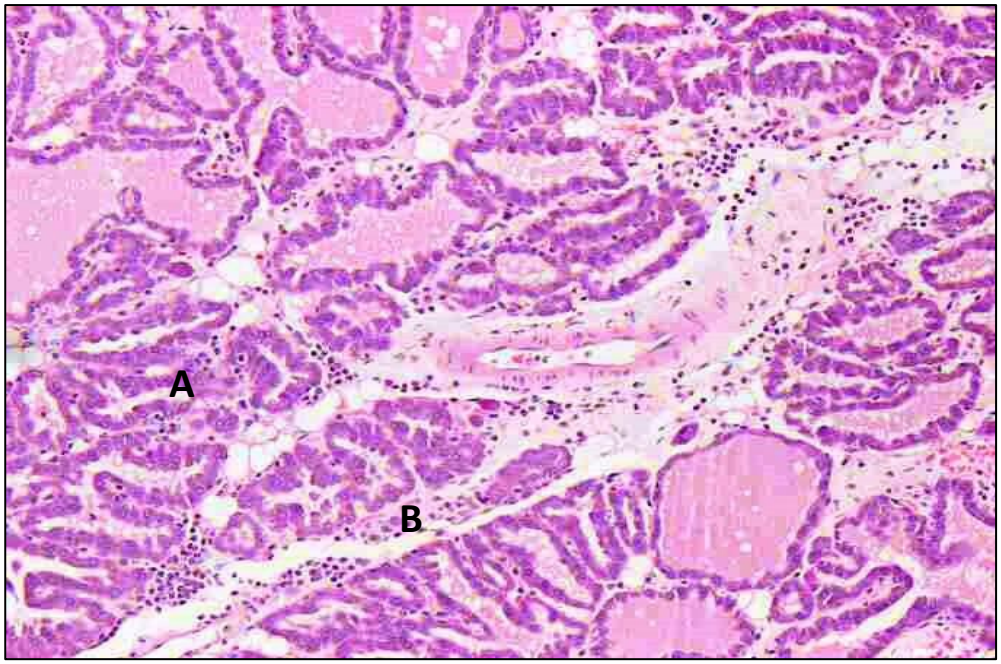


Figure 7.3: Mammary tissue specimen from lactating mouse immunized with anti IL6 antibody via intraperitoneal route and challenged using non typeable *S. aureus* 83. Mammary glands (A) Infiltration of acute inflammatory cells in perilobular connective tissue (B). Level 1 category inflammation. H&E x 200mag.

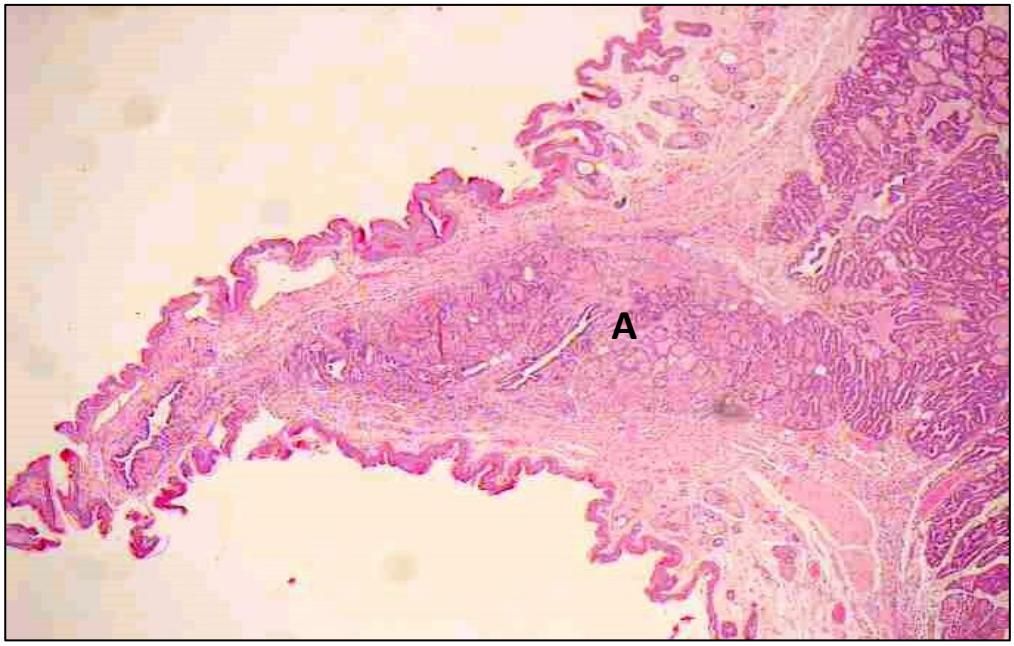
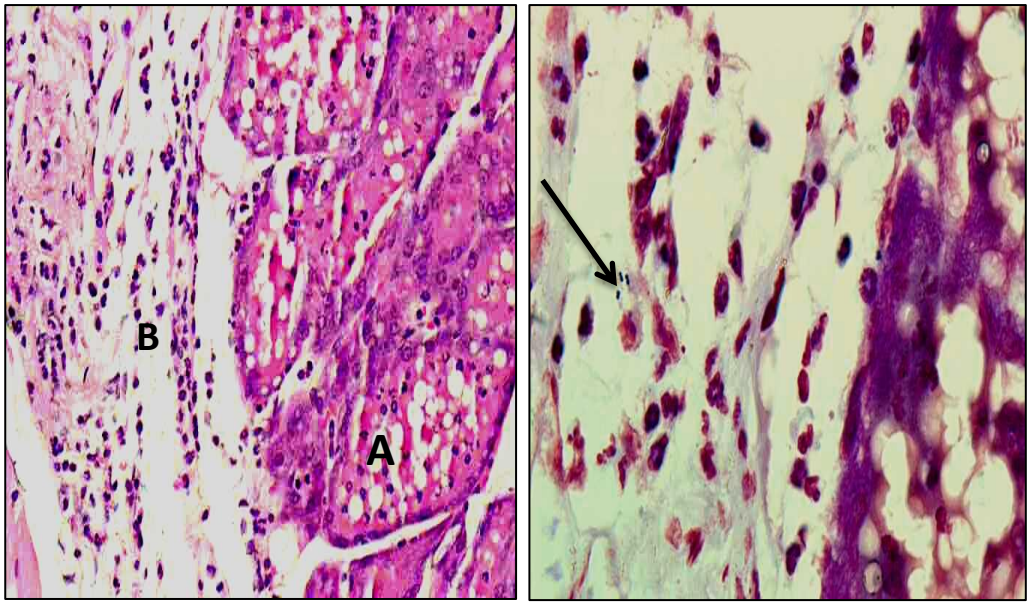


Figure 7.4: Mammary tissue specimen from lactating mouse immunized with anti IL6R β antibody via intraperitoneal route and challenged using non-typeable *S. aureus* 83. Mild inflammatory cell infiltrate in periductal connective tissue (A). Level 1 category inflammation. H&E x 40mag.



7.5(a) 7.5 (b)

Figure 7.5: Mammary tissue specimen from lactating mouse immunized with anti IL6R β antibody via intraperitoneal route and challenged using non-typeable *S. aureus* 83 from sample 11 (Figure 7.4). 7.5(a) Small numbers of inflammatory cells in intralobular (A) and perilobular site (B). Level 1 category inflammation. H&E x 400 mag. 7.5(b) Gram positive bacteria (Arrow) in inflammatory material Gram Twort x1000mag.

Chapter 8: Evaluation of the immunogenicity and protective potential of planktonic versus biofilm-based whole cell vaccines against bovine mastitis using the mouse mastitis model

8.1 Introduction

Staphylococcus aureus is one of the prominent etiological agents of clinical, subclinical, persistent and chronic forms of mastitis in cows (Clutterbuck et al., 2007; Middleton, 2008; Pereira et al., 2011). It is normally present in almost all the external surfaces of cow (Roberson et al., 1998) and udder is the most preferred site for colonization of *S. aureus* in dairy cows (Jorgensen et al., 2005). Upon entry into the mammary gland *S. aureus* cells adhere to the epithelial lining of mammary gland. This is followed by formation of multilayered clusters of *S. aureus* cells which is enclosed by a slimy extracellular matrix known as biofilm (Melchior et al., 2006). Formation of biofilm in the mammary gland makes treatment of mastitis difficult as *S. aureus* in biofilm does not respond to antimicrobial therapy resulting in chronic mastitis (Cucarella et al., 2004).

The current conservative therapy of mastitis includes the use of antimicrobial agents. Despite scheduling treatment regimens to prolong the availability of appropriate antibiotics for an extended period of time in the mammary gland (Erskine, 2001), the cure rates of mastitis particularly for *S. aureus* infections have not been very satisfactory (Timms, 2001). This has further been complicated by the biofilm forming ability of the pathogen as the pathogen in biofilm is protected from the effect of antimicrobial agents. *S. aureus* growing in a biofilm expresses different genes than the *S. aureus* growing in planktonic form (Beloin et al., 2004; Waite et al., 2005; Yao et al., 2005; Resch et al., 2006; Shemesh et al., 2007). Moreover, *S. aureus* carrying biofilm-associated genes such as *ica* types, *bap*, *agr* types have increased potential to induce (Baselga et al., 1993) and develop recurrent infections of mammary gland (Cucarella et al., 2004). As such, there is a serious need to develop a suitable vaccine to prevent *S. aureus*-associated bovine mastitis. While formulating an effective vaccine, it is important to consider the different antigens which are expressed in biofilm form of growth of *S. aureus.*

A wide variety of extracellular virulence antigens of *S. aureus* have been reported to be associated with biofilm formation. These mediators include poly-N acetyl glucosamine (PNAG) or polysaccharide intercellular adhesion (PIA) [Vuong et al., 2004], surface associated proteins viz. FnBPA, FnBPB, ClfA, ClfB, CNA, bap, ProteinA, SasG and bone sialo binding protein (BBP) [Patti et al., 1992; Mc Devitt et al., 1994; Tung et al., 2000; Cucarella et al., 2001; Corrigan et al., 2007; O'Neill et al., 2008; Merino et al., 2009; Vergara-Iriqaray et al., 2009]. Extracellular DNA (eDNA) has also been found to be associated with biofilm formation of *S. aureus* (Rice et al., 2007; Izano et al., 2008).

Various studies have been conducted to test the immunogenicity potential of some of these molecules. However, when tested for their protective potential as "single antigen" vaccines, only partial protection measured by passive transfer of antigen specific antibodies or as immunogens (Mamo et al, (1994; Maitran et al, 2004; Zhou et al, 2006) A study conducted by Cywes-Bentley et al (2013) demonstrated that mice immunized with antibodies against PNAG protected them from *S. aureus* associated localized and systemic infections. However, slime associated antigenic complex (SAAC) also known as PNAG, failed to prevent intramammary infection in cows, only reduced the multiplication of *S. aureus* in the mammary gland (Prenafeta et al., 2010). FnBpA and Protein A have been found to provide partial protection to mice infected with *S. aureus* systemic infection (Zhou et al., 2006; Kim et al., 2010; Kim et al., 2012). Similarly, vaccination of mice with the CNA-FnBP proteins reduced the post-challenge mortality (Zhou et al, 2006) whereas dairy heifers vaccinated with these proteins alone or in combination were not effective in countering challenge with the parent strain.

Due to involvement of multiple mediators in the development of biofilm by *S. aureus*, a vaccine containing a single or two antigens as vaccine candidates can only provide partial protection against biofilm associated *S. aureus* infections. Literature indicates that gene expression of a bacterial pathogen residing in biofilm is different from the bacteria from its planktonic form (Prignent-Combaret et al., 1999). Moreover, a study has demonstrated that host immune response to planktonic form of *S. aureus* is different from its biofilm counterpart (Thurlow et al, 2011). The authors

found that *S. aureus* in biofilm attenuate the traditional antibacterial host immune response and reduces the production of cytokine or chemokines and thus altering the host immune system. We hypothesize that a vaccine composed of resident *S. aureus* cells from biofilm will provide better immune response and protection against *S. aureus*-associated mastitis due to reduction in the strength of biofilm forming ability. Based on this hypothesis, the immunogenicity and protective potential of formalin killed *S. aureus* planktonic versus biofilm vaccines were carried out using mouse mastitis model.

8.2 Material and Methods

8.2.1 Immunogenicity and protective potential of planktonic versus biofilm *S. aureus*-based vaccines

8.2.1.1 Bacterial strain

BOAISRF *S. aureus* 51, a clinical CP 8 positive and a strong biofilm producer (genotype shown in Table 8.1) was used in this study. This strain was the most virulent encapsulated strain as judged by extent of mammary tissue damage and the number of *S. aureus* isolated from mammary gland post-infection. The strain was selected from a collection of 154 bovine mastitis-associated *S. aureus* strains obtained from Victoria and Queensland, Australia. The details of the genotypic and/or phenotypic characteristic of this strain determined in this project embodying this PhD thesis are listed in Table 8.1.

The parental strain (BOAISRF *S. aureus* 51) was used in the challenge experiment to determine the protective potential of the planktonic versus the biofilm *S. aureus* vaccines.

8.2.1.2 Animal ethics approval

A total of 96 numbers of 3days pregnant Balb/c mice were obtained from the Animal Resources Centre, Perth, Western Australia and used for this study. All animal experiments were carried out with the approval of Curtin University's Animal Ethics Committee (Approval No: AEC_2013_24) ensuring compliance with the Western Australian Animal Welfare Act, 2002.

Table 8.1: Phenotypic/genotypic characteristics of BOAISRF *S. aureus* 51 strain used in development of planktonic and biofilm vaccines

Phenotypic/ genotypic characteristics	
Biofilm formation	Strong
Capsular Polysaccharide type	CP8
Presence of Biofilm associated genes	*ica*A, *ica*D, *agr* typeIII
Presence of MSCRAMMS genes	*spa, can, clf*A, *clf*B, *α-isd*A, *α-isd*B, *sdr*D, *sdr*E
Presence of toxin genes	*hla, hlb, seh*

8.2.1.3 Preparation of vaccines

24 well flat bottom with lid tissue culture plates (Sarstedt Australia Pty Ltd, SA) were filled with 1.5 ml of 1% glucose enriched nutrient broth. Single colony of overnight grown BOAISRF 51 strain was inoculated into each well. Plates were incubated at 37º C for 24 h with gentle shaking at 80 rpm. The supernatant (Planktonic vaccine) was collected carefully by using a pipette and centrifuged at 10,000 x g for 10 min. The biofilm was collected by scooping out with sterile mini spatula (LEAP Biosciences) and washed using 1ml of sterile PBS. Both the planktonic and biofilm forming cells were washed for five times at 10,000 x g for 10 min by using PBS. Finally the cells were suspended in 5 ml of PBS and stored at 4ºC to prevent growth. Tenfold dilutions up to 10^{11} was performed for both the planktonic and biofilm suspensions and 100 µl from each dilution was plated on Baird Parker agar media plates in triplicates by using spread plate method. The plates were incubated at 37ºC for 48 h followed by determination of colony counts of *S. aureus*. To each 3 ml of planktonic/biofilm suspensions, 2 ml of 3% formalinized PBS was added and allowed to remain at room temperature for a period of 24 h. The bacterial suspensions were then subjected centrifugation at 10,000 x *g* for 20 min and washing with PBS five times. Finally the pellet was suspended in 5ml of sterile PBS. Based on the growth curve, the desired colony forming units of BOAISRF *S. aureus* 51 in both planktonic and biofilm form were adjusted to 10^7 CFU/0.05 ml, 10^8 CFU/0.05 ml and 10^9 CFU/0.05 ml.

8.2.1.4 Preparation of bacterial inocula for challenge

BOAISRF *S. aureus* 51 was grown on MH agar plates at 37°C for 18h. The pure colonies were washed from the plates using 20 ml of isotonic saline and suspended in isotonic saline to give a final viable bacterial count of 4 x 10^{11} ml^{-1} (Anderson and Chandler, 1975).

8.2.1.5 Immunization protocol

A total of 96 mice were divided into 8 groups comprising 12 mice in each group. The details of the groups are presented in Table 8.2. All the four test groups of mice were vaccinated with 3 doses of 0.1 ml of killed whole cell vaccines. 0.05 ml of vaccine containing an equivalent of 10^7, 10^8 and 10^9 CFUs/mL was mixed in equal proportion of alum-based adjuvant such as Imject Alum (Thermo Fisher Scientific) and injected to the mice on days 0, 7 and 14 day, respectively using s/c and i/mam routes. Mice in the control groups were vaccinated with 0.1 ml of sterile PBS on 0, 7 and 14 days using the same routes as that used in test mice. For i/mam inoculation, mice were anaesthetised with intraperitoneal (i.p.) dose of 100 mg ketamine kg−1 and 10 mg xylazine kg−1, put on their back under a binocular and the teats and the surrounding area were disinfected with 70% ethanol. The hind teat, numbered R5, was held with fine forceps and the duct orifice was located. A volume of 0.1 ml of killed vaccines was injected through the orifice using 31 gauge blunt needles. The needle was not inserted more than four millimetres deep into the canal. The control mice were inoculated with sterile PBS. The mice were monitored twice a day. Six mice from each group were euthanized on 28^{th} day of vaccination and blood and spleen were sampled for detection of antigen-specific antibodies in serum and level of Interferon gamma (IFN-γ) in splenocyte supernatants. Remaining 48 mice were used for challenge experiment.

8.2.1.6 Detection of antigen-specific antibodies in serum

Antigen-specific antibody isotype analysis of sera samples of mice immunised with the planktonic or biofilm vaccine was carried out prechallenge. The levels of anti-*S. aureus* IgG, IgG_1 and IgG_{2a} were determined using an indirect ELISA (Chapter 5, Section 5.2.3.1).

Table 8.2: Experimental design for determination of immunogenicity and protective potential of planktonic versus biofilm vaccines (n=96)

Sl. No	Group	Route of administration	Number of mice
1	Planktonic test	s/c	12
2	Planktonic control	s/c	12
3	Planktonic test	i/mam	12
4	Planktonic control	i/mam	12
5	Biofilm test	s/c	12
6	Biofilm control	s/c	12
7	Biofilm test	i/mam	12
8	Biofilm control	i/mam	12

8.2.1.7 Estimation of Interferon gamma (IFN-γ) in antigen stimulated splenocyte supernatants

Methods for the stimulation of splenocytes cultured *in vitro* and collection of splenocyte supernatants have been detailed in Chapter 2 Section 2.5. Estimation of IFN-γ, as an indirect indicator of cell mediated immunity (CMI) was carried out for the vaccinated and control groups of mice. This was accomplished using ab46081 IFN gamma Mouse ELISA kit (Abcam®) following manufacturer's instructions.

8.2.1.8 Challenge experiment

A total of 48, three days pregnant Balb/c mice which were vaccinated on day 0, 7 and 14 days (Described in Chapter 8, Section 8.2.1.5) were challenged on the 28th day of post vaccination. The lactating mice (5-15 days) from both test and control groups were challenged in both L5 and R5 mammary glands using 50 µl (CFU $2x10^{10}$) of homologous strain of BOAISRF *S. aureus.* Mice were challenged via intra-mammary route using thirty one gauge needles (Chapter 6, Section 6.2.4.4). The mice were observed twice daily for morbidity and mortality until 5 days post-challenge. Percent protection against mouse mastitis was recorded and surviving mice euthanized after sampling blood and mammary glands.

8.2.1.8.1 Clinical observation

The mammary glands of mice were observed for clinical signs of mastitis including redness, swelling and discolouration of mammary gland and extrusion of exudate with or without squeezing of the mammary gland (Chapter 6, Section 6.2.5.1).

8.2.1.8.2 Bacterial load study of mammary glands

The L5 mammary glands from control and test mice were collected and subjected to bacterial load study (Chapter 6, Section 6.2.5.2.1)

8.2.1.8.3 Histological observation

R5 mammary glands were collected from both control and test mice for histopathological examination (Chapter 6, Section 6.2.5.3.1)

8.2.1.9 Statistical analysis

To compare the antigen specific antibody levels, Interferon gamma (IFN-γ) levels and Log average number of bacteria (CFU) recovered from mammary glands between groups of mice immunized with planktonic and biofilm *S. aureus* vaccines, student's *t*-test was performed. Statistical significance was set at $p < 0.05$.

8.3 Results

8.3.1 Immunogenicity trial using planktonic versus biofilm based vaccines

8.3.1.1 Detection of antigen-specific antibody classes/isotypes in sera samples of mice

The serum IgG and IgG1 antibody titres of mice immunised with the planktonic *S. aureus* vaccine using subcutaneous route were significantly greater than the titres induced by vaccination using intramammary route (Figure 8.1A and 8.1B).

The serum IgG_{2a} and IgG antibody titres of mice immunised with the biofilm *S. aureus* vaccine using intramammary route were significantly higher than the mice vaccinated by biofilm *S. aureus* vaccine using subcutaneous route (Figure 8.2A and 8.2B).

Figure 8.1A: Detection of antigen-specific antibody isotypes of mice vaccinated with planktonic killed *S. aureus* vaccine by s/c route

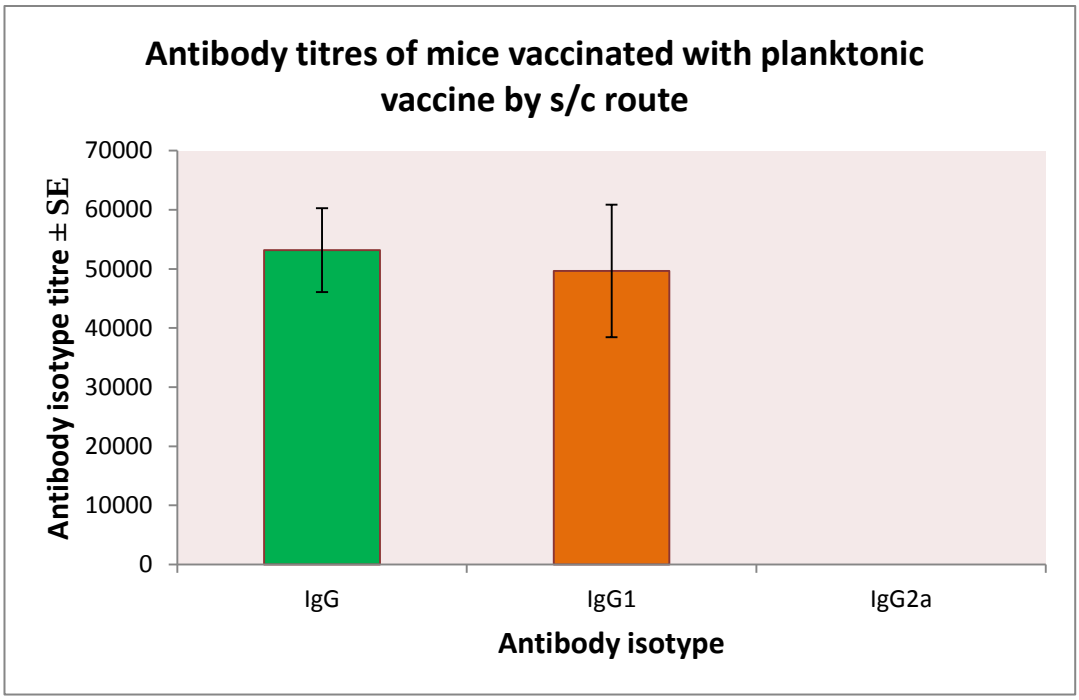


Figure 8.1B: Detection of antigen-specific antibody isotypes of mice vaccinated with planktonic killed *S. aureus* vaccine by i/mam route

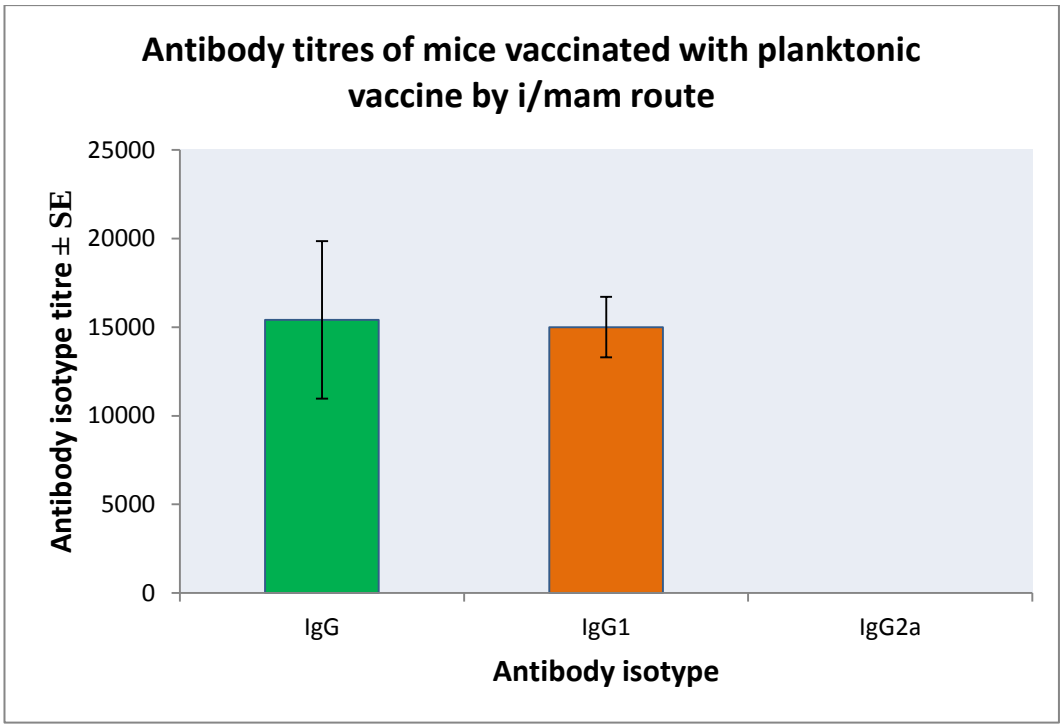


Figure 8.2A: Detection of antigen-specific antibody isotypes of mice vaccinated with biofilm killed *S. aureus* vaccine by s/c route

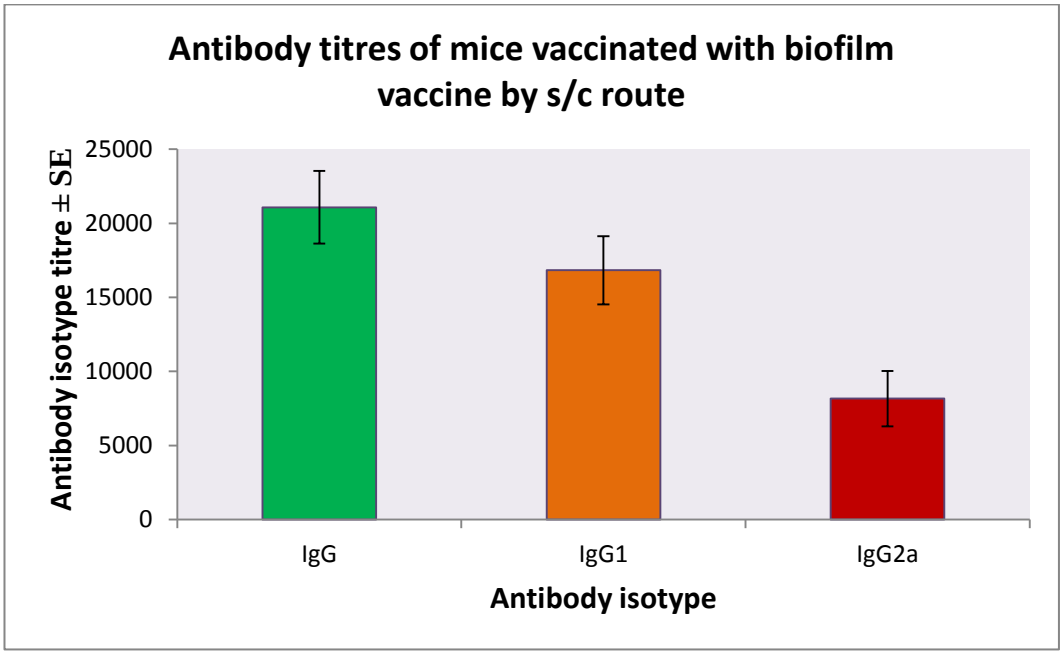


Figure 8.2B: Detection of antigen-specific antibody isotypes of mice vaccinated with biofilm killed *S. aureus* vaccine by i/mam route

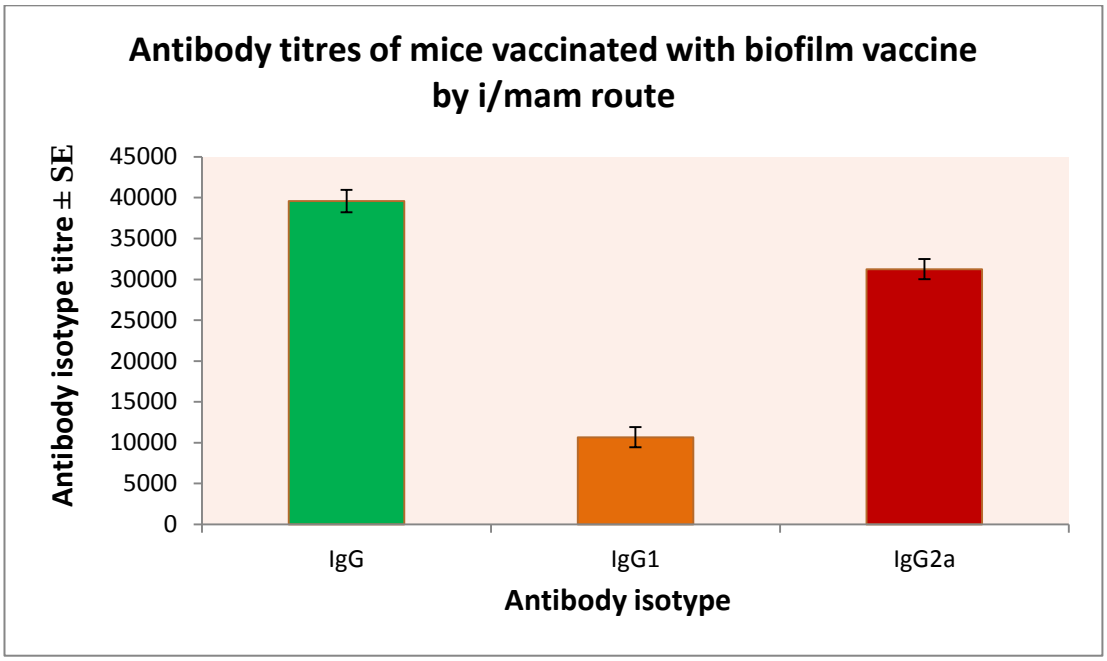
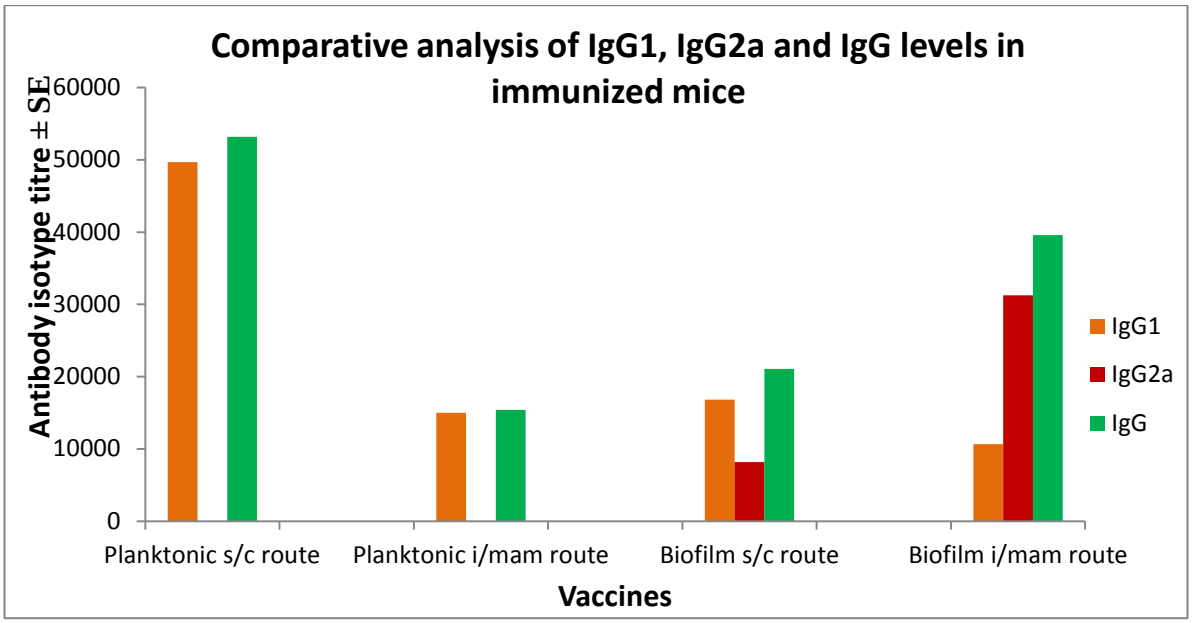


Figure 8.3: Comparison of levels of antigen-specific antibody isotypes of mice vaccinated with planktonic and biofilm killed *S. aureus* vaccine by using subcutaneous and intramammary routes



8.3.1.2 Detection of Interferon gamma (IFN-γ) levels in splenocyte supernatants

Mice vaccinated with *S. aureus* planktonic and biofilm vaccines using both s/c and i/mam routes produced variable levels of IFN-γ when splenocytes were stimulated with killed planktonic and biofilm BOAISRF *S. aureus* 51. Mice vaccinated with *S. aureus* planktonic vaccine via s/c and i/mam routes produced 48.2±15.61 pg/ml and 19.06±12.06 pg/ml IFN-γ, respectively when the splenocytes were stimulated with killed planktonic *S. aureus*. However, splenocytes stimulated with ConA produced 6220±34.16 pg/ml of IFN-γ (Figure 8.4).

The splenocytes of mice vaccinated with *S. aureus* biofilm vaccine using s/c and i/mam routes produced 460.87±148.69 pg/ml and 4259.33±367.11 pg/ml of IFN-γ, respectively when the splenocytes were stimulated with killed biofilm *S. aureus*. The splenocytes stimulated with ConA produced 6166.67±47.73 pg/ml of IFN-γ (Figure 8.5).

8.3.2 Protection study against planktonic and biofilm based vaccines

8.3.2.1 Clinical observation of mammary glands of challenged mice for mastitis

The control groups of mice showed prominent signs of mastitis in the infected glands. All the test mice showed varying degrees of changes including redness, swelling, and discolouration of mammary gland, exudate and morbidity (Table 8.3, Figure 8.6).

Figure 8.4: Concentration of IFN-γ produced by killed planktonic *S. aureus* stimulated splenocytes of mice immunized with planktonic *S. aureus* vaccine using different routes

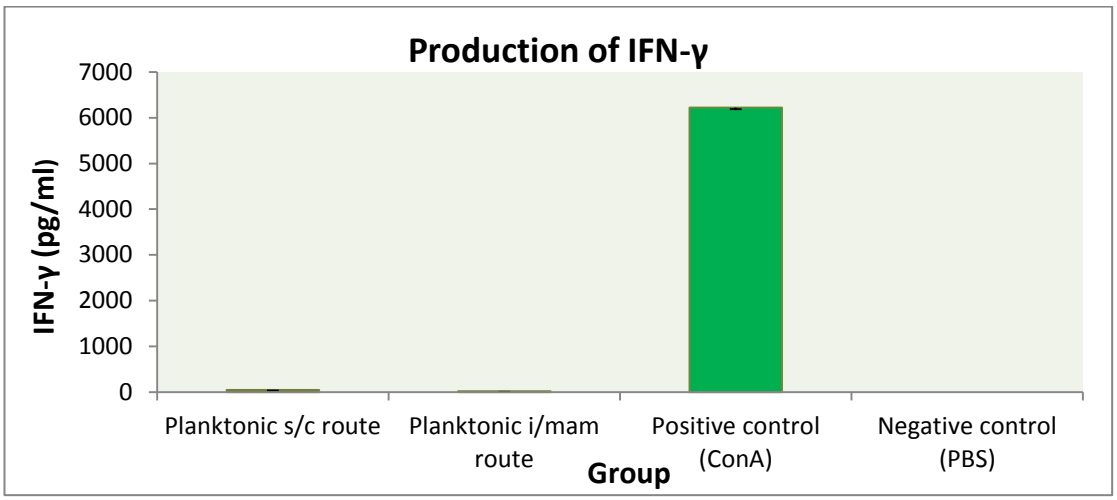


Figure 8.5: Concentration of IFN-γ produced by killed biofilm *S. aureus* stimulated splenocytes of mice immunized with biofilm *S. aureus* vaccine using different routes

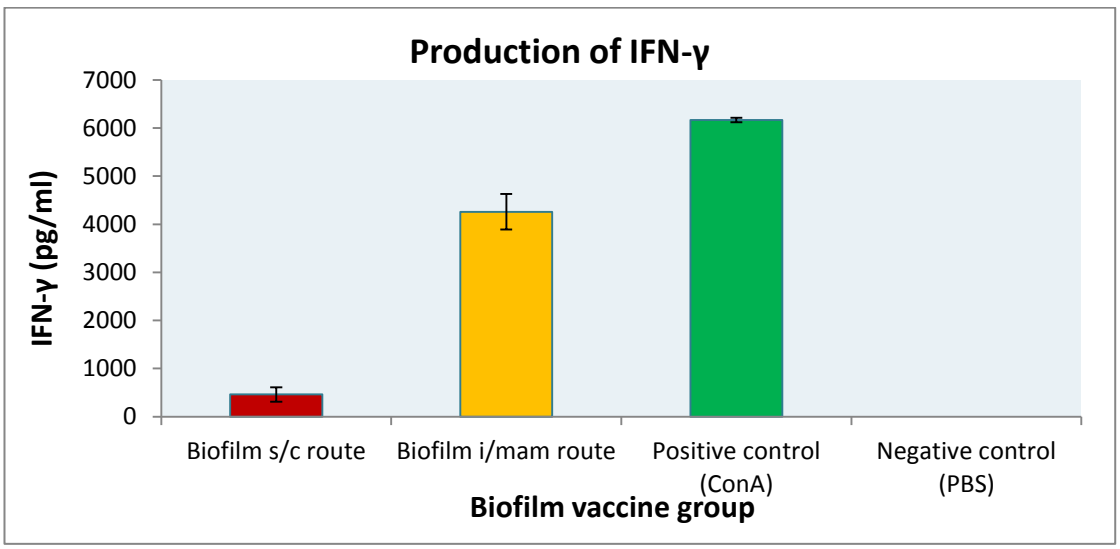


Table 8.3: Clinical signs observed in different groups of mice immunized with planktonic and biofilm *S. aureus* vaccines (Observations up to 5 days post cahllenge)

Level of clinical signs* observed in mice injected with Planktonic and biofilm vaccines						
Sl. no	**Vaccination group**	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**
1	Planktonic vaccine (s/c route)	+	++	++	++	++
2	Planktonic vaccine (i/mam route)	+	++	++	++	++
3	PBS control for planktonic s/c route	++	+++	+++	+++	+++
4	PBS control for planktonic i/mam route	++	+++	+++	+++	+++
5	Biofilm vaccine (s/c route)	+	++	++	++	++
6	Biofilm vaccine (i/mam route)	0	+	+	+	+
7	PBS control for biofilm s/c route	++	+++	+++	+++	+++
8	PBS control for biofilm i/mam route	++	+++	+++	+++	+++

*Clinical features include redness, swelling, and discolouration of mammary gland, exudate, morbidity and mortality. Grade scores compare observed features to the most severe changes: 0 - no macroscopic changes, + low grade, ++ medium grade, +++ severe grade

Figure 8.6: Observation of clinical symptoms of mastitis in mammary glands of mice vaccinated with killed whole cell planktonic and biofilm vaccine

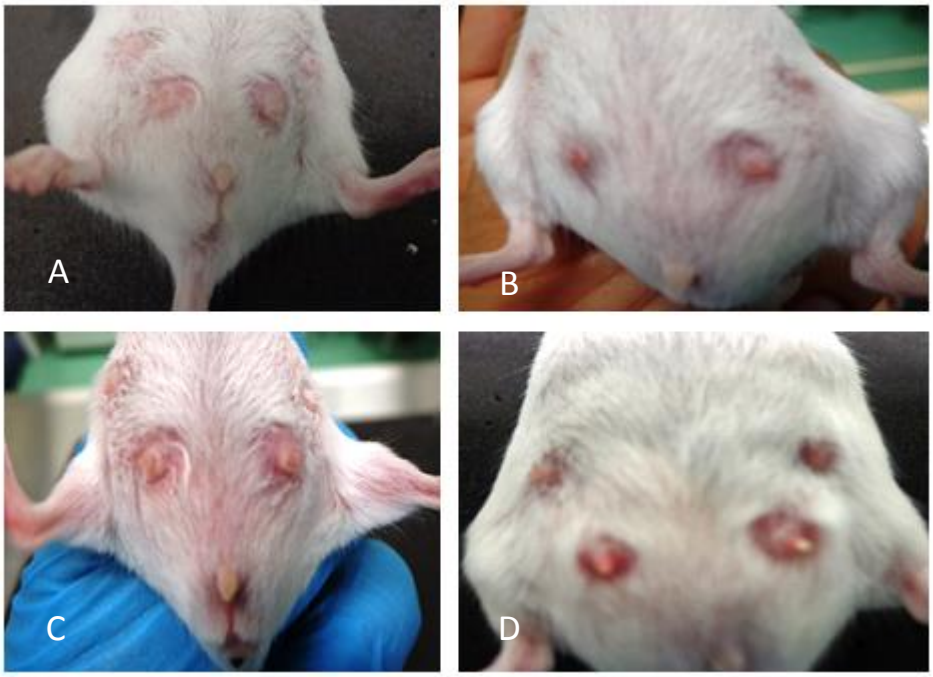


Fig 8.6: Mammary glands of mice showing various levels of clinical symptoms including redness, swelling, discolouration and exudate in mammary gland, exudate. A: Mammary gland of mouse immunized by killed whole cell biofilm vaccine via i/mam route after 24 h of post challenge. No visible signs of mastitis. Mammary gland appears normal, Grade score o. B: Mammary gland of mouse immunized by killed whole cell biofilm vaccine via i/mam route after 120 h of post challenge. Grade score + low grade. C: Mammary gland of mouse immunized by killed whole cell planktonic vaccine via s/c route after 120 h of post challenge. Grade score ++ medium grade. D: Mammary gland of mouse immunized by PBS after 120 h of post challenge. Grade score +++ severe grade.

8.3.2.2 Bacterial load study of mammary glands of challenged mice

The bacteriological load study demonstrated that mice vaccinated with planktonic *S. aureus* vaccine had a bacterial load of 6.58±0.24 versus 6.63±0.38 CFU *S. aureus* in the mammary glands immunized by s/c and i/mam routes, respectively. The control mice for planktonic *S. aureus* vaccine group showed 6.85±0.049 and 7.68±0.05 CFU of *S. aureus* in their mammary glands when mice were injected by s/c and i/mam routes, respectively. The mice vaccinated with biofilm *S. aureus* vaccine showed

5.64±0.05 and 5.57±0.01 CFU of *S. aureus* in the mammary glands when vaccinated by s/c and i/mam routes, respectively. The control mice in biofilm *S. aureus* vaccine group showed 6.80±0.48 and 7.63±0.08 CFU of *S. aureus* when vaccinated by s/c and i/mam routes, respectively. The details of the bacterial load study is presented in Table 8.4.

8.3.2.3 Histopathological observation of mammary glands of mice challenged with *S. aureus* 51 strain

Histopathological grade observed in the mammary tissue are shown in Table 8.4 and Figures 8.7- 8.14. All the mice from groups including control, planktonic vaccine (s/c and i/mam routes) and biofilm (s/c route) demonstrated level 3 histopathological changes in mammary glands. However, 6 mice vaccinated with biofilm (i/mam route) vaccine showed histopathological grading as 1, 2, 2, 2, 2, 1 in M1 to M6, respectively.

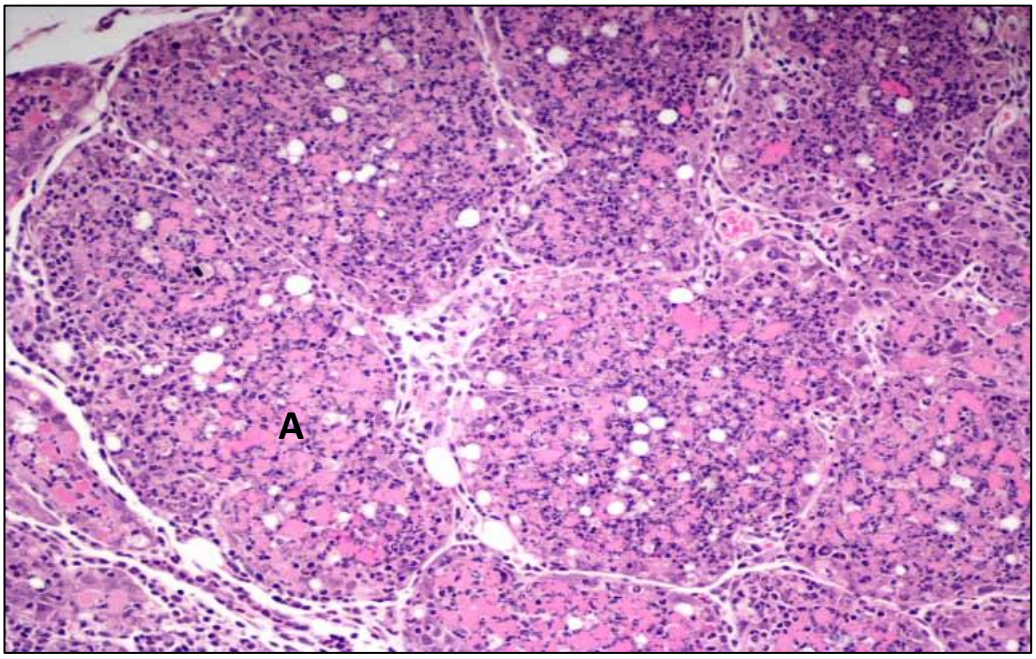


Figure 8.7: Mammary tissue specimen from lactating mouse immunized with killed *S. aureus* Planktonic vaccine (s/c route) and challenged using strong biofilm forming *S. aureus* 51. Interlobular acute inflammatory infiltration with cellular necrosis (A). Level 3 category inflammation. H&E x 400mag.

Table 8.4: Detection of bacterial load and histopathological changes in the mammary glands of mice vaccinated with *S. aureus* planktonic and biofilm vaccines

Sl. No	Vaccine group	Total number of mammary glands investigated	Log average number of bacteria (CFU) recovered from mammary glands	Grades for histopathological changes					
				M1	M2	M3	M4	M5	M6
1	Planktonic s/c route	6	6.58±0.24	3	3	3	3	3	3
2	Planktonic i/mam route	6	6.63±0.38	3	3	3	3	3	3
3	PBS control for planktonic s/c route	6	7.68±0.05	3	3	3	3	3	3
4	PBS control for planktonic i/mam route	6	6.85±0.049	3	3	3	3	3	3
5	Biofilm vaccine (s/c route)	6	5.64±0.05	3	3	3	3	3	3
6	Biofilm vaccine (i/mam route)	6	5.57±0.01	1	2	2	2	2	1
7	PBS control for biofilm s/c route	6	6.80±0.48	3	3	3	3	3	3
8	PBS control for biofilm i/mam route	6	7.63±0.08	3	3	3	3	3	3

M*= Mammary gland

In paired *t* test between biofilm vaccine (s/c and i/mam routes) with planktonic vaccine (s/c and i/mam routes) demonstrated significant difference (< 0.05) in bacterial load and histopathological grades of mammary tissue lesions between biofilm vaccine (i/mam route) with both s/c and i/mam routes of planktonic vaccine. Biofilm vaccine (s/c) route although demonstrated significant difference in bacterial load compared to planktonic vaccine by either s/c or i/mam route, but no significant difference was detected in respect to tissue damage.

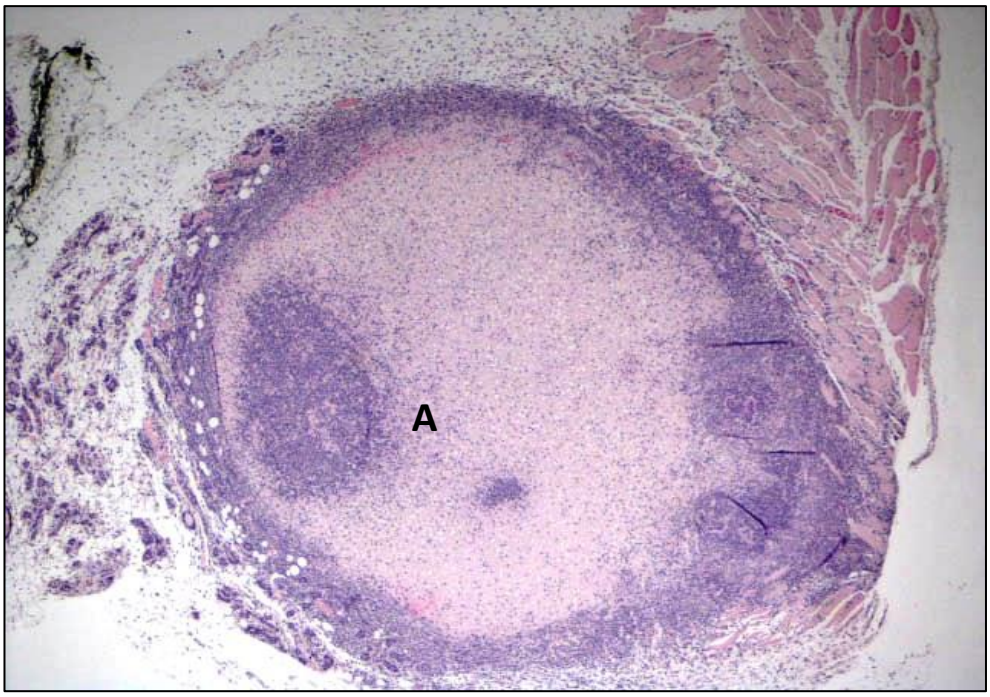


Figure 8.8: : Mammary tissue specimen from lactating mouse immunized with killed *S. aureus* Planktonic vaccine (i/mam route) and challenged using strong biofilm forming *S. aureus* 51. Inflammatory abscess shows inflammatory exudate in fibrinous capsule. Level 3 category inflammation. H&E x 40mag.

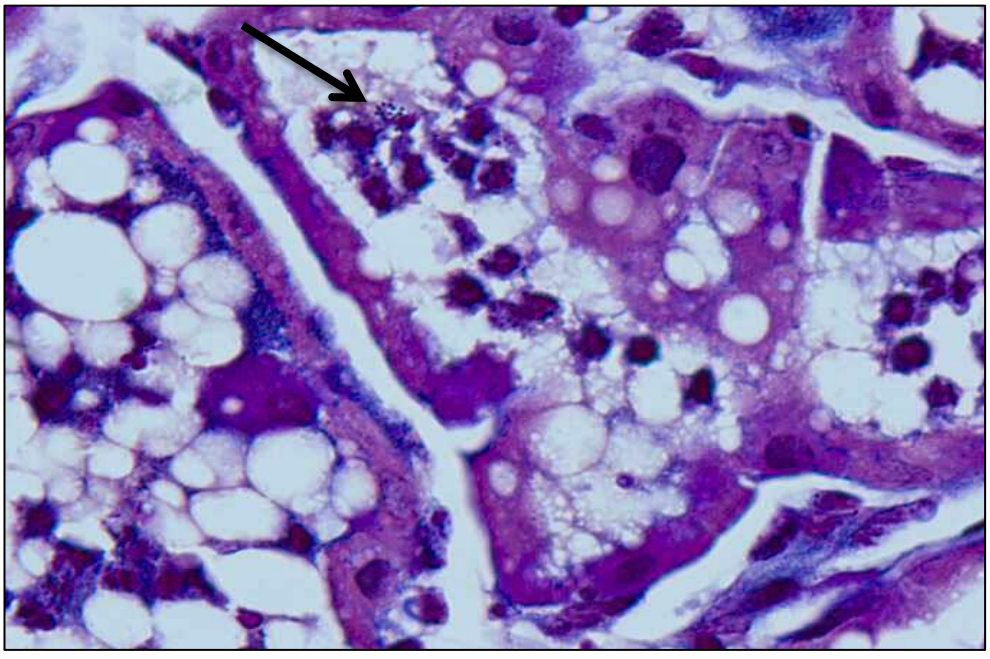


Figure 8.9: Mammary tissue specimen from lactating mouse immunized with killed *S. aureus* Planktonic vaccine (i/mam route) and challenged using strong biofilm forming *S. aureus* 51. Gram positive bacteria within interlobular inflammatory infiltrate (Arrow). Gram Twort x1000mag.

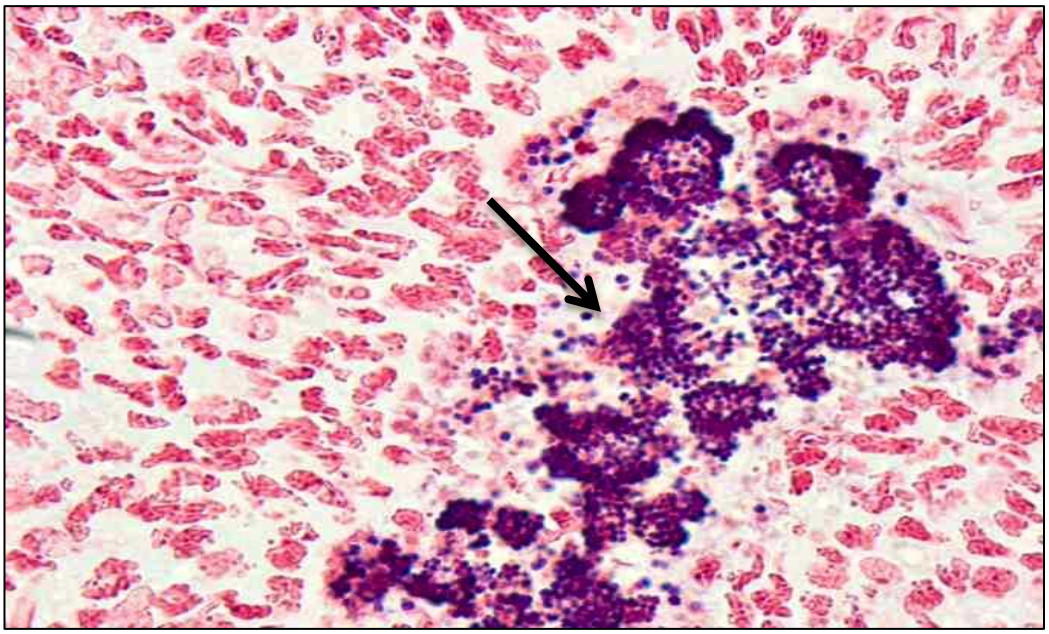


Figure 8.10: Mammary tissue specimen from lactating mouse injected with PBS (i/mam route) and challenged using strong biofilm forming *S. aureus* 51. Gram positive bacteria (Arrow) and associated inflammatory cell exudate. Gram Twort x1000mag.

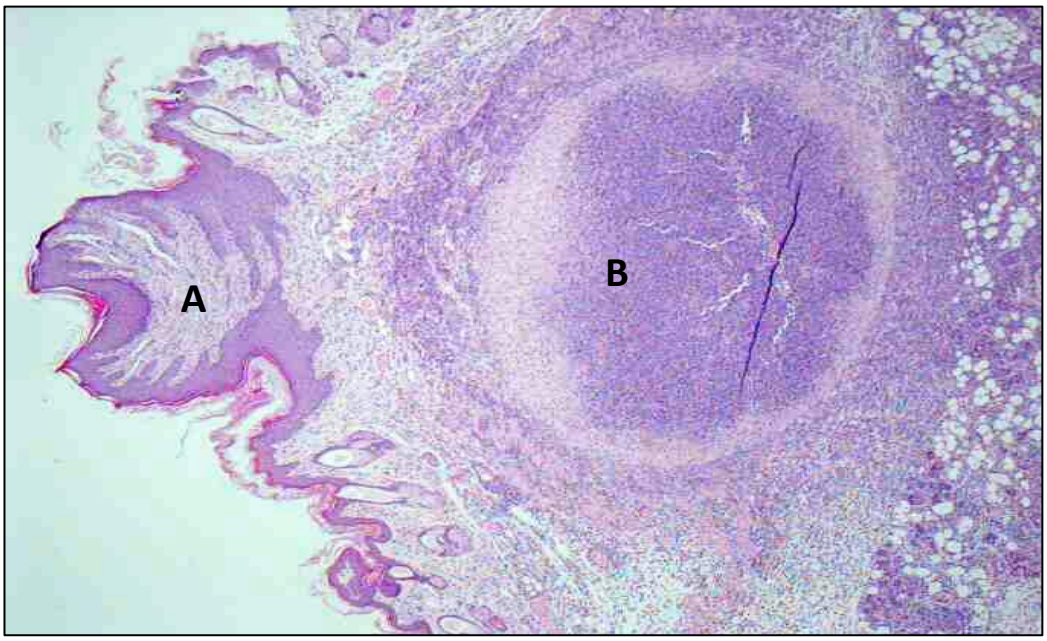


Figure 8.11: Mammary tissue specimen from lactating mouse immunized with killed *S. aureus* biofilm vaccine (s/c route) and challenged using strong biofilm forming *S. aureus* 51. (A) Mammary teat (B) Intramammary abscess shows central liquefactive necrosis and fibrinous capsule. Level 3 category inflammatory lesion. H&E x 40mag.

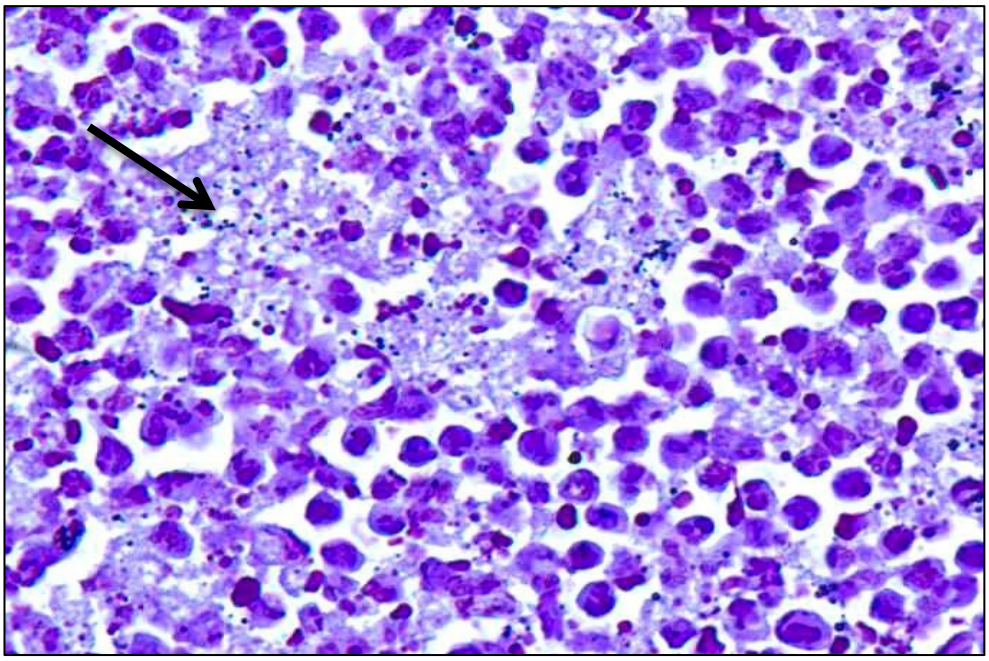


Figure 8.12: Mammary tissue specimen from lactating mouse immunized with killed *S. aureus* biofilm vaccine (s/c route) and challenged using strong biofilm forming *S. aureus* 51. Gram positive bacteria (Arrow) within suppurative inflammatory material. Gram Twort x1000mag.

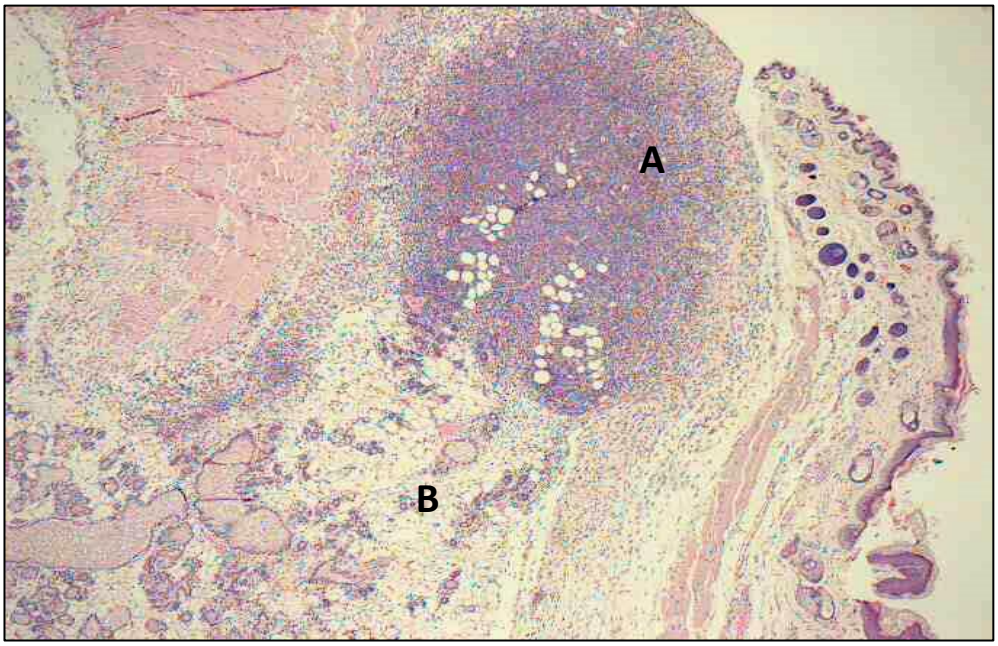


Figure 8.13: Mammary tissue specimen from lactating mouse immunized with killed *S. aureus* biofilm vaccine (i/mam route) and challenged using strong biofilm forming *S. aureus* 51. (A) Inflammatory lesion of mixed cellularity includes acute inflammatory macrophages and fibroblasts (B). Level 2 category inflammation. H&E x 40mag.

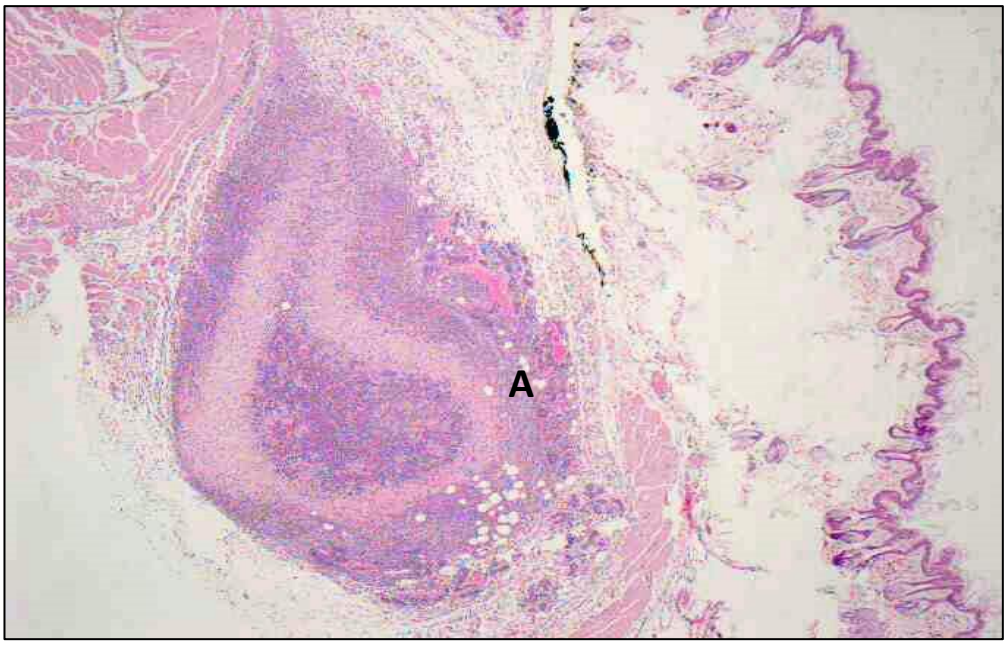


Figure 8.14: Mammary tissue specimen from lactating mouse injected with PBS and challenged using strong biofilm forming *S. aureus* 51. Level 3 category inflammation. Abscess in mammary tissue with tissue necrosis (A). H&E x 40mag.

8.4 Discussion

S. aureus is frequently associated with formation of biofilm in the mammary gland making antibiotic therapy of bovine mastitis difficult (Melchior et al., 2006). The *S. aureus* residing in biofilm do not respond as well to antimicrobial agents and resist host immune mechanism (Gil et al., 2014). Upon entry into the mammary gland *S. aureus* attaches to the epithelial lining of mammary gland using specific receptors and starts colonizing. At this stage, these planktonic *S. aureus* cells are either eliminated by immune system of host or the cells end up developing biofilm by attaching to the extracellular matrix proteins of host (Harro et al, 2010). Biofilm of *S. aureus* is composed of two major components: extracellular matrix and *S. aureus* cells within extracellular matrix. While formulating a vaccine development against *S. aureus*-associated infections, the growth form of the pathogen needs to be considered as there is difference in gene expression, cellular attachment, cellular regulatory circuits and virulence of planktonic and biofilm forms of growth of bacterial pathogens (Prignent-Combaret et al., 1999; Serra et al., 2008; Babra et al., 2014 unpublished data). To the best of our knowledge the majority of the biofilm vaccine trials have included extracellular matrix itself or the associated protein of

extracellular matrix to formulate a vaccine and study its protective potential in mouse model (Maira Litran et al., 2005; Prenafeta et al., 2010; Skurnik et al., 2010; Gil et al., 2014). Use of slime-associated antigenic complex (SAAC) of *S. aureus* have been reported to protect *S. aureus* localized and systemic infections in mice (Cywes-Bentley et al., 2013) but failed to protect intramammary infection in cows, only leading to a reduction in the multiplication of *S. aureus* in the mammary gland (Prenafeta et al., 2010). PNAG being immune evasion molecule of *S. aureus* and component of polysaccharide intercellular adhesion of biofilm has been targeted as a potential vaccine candidate (Mc Kenney et al., 1998; Maira Litran et al., 2005; Perez et al., 2009; Gening et al., 2010). Maira Litran et al (2005) demonstrated that deacetylated PNAG (dPNAG) is more efficient than the acetylated (90%) form of PNAG as the acetylated form gets released into the suspension while the dPNAG retains on cell surface of bacteria (Cerca et al., 2007). However, vaccine studies using PNAG as a potential vaccine candidate though reduced the number of *S. aureus* at the site of infection but failed to provide complete protection against *S. aureus* associated infections (Middleton, 2008). A recent study to detect the protective immune response of biofilm matrix exoproteome was carried out in CD1 mice using mesh-associated biofilm infection model (Gil et al., 2014). The study demonstrated reduction in the colonization of *S. aureus* in kidney and liver of mice, but failed to protect infection in the mice.

Our approach was to formulate a killed whole cell biofilm vaccine based on the presence of *ica* genes promoting biofilm formation. However, the fact that S. *aureus* without *ica* genes is also capable of producing biofilm, potentially due to the presence of various surface proteins of *S. aureus* (Cucarella et al., 2001). Moreover, to the best of our knowledge, an approach to formulate a killed while cell vaccine using the resident *S. aureus* cells from biofilm has not been studied using mouse mastitis model. It was decided to formulate a killed whole cell vaccine using *S. aureus* resident in the biofilm and evaluate its immunogenicity and protective potential using mouse mastitis model. This study has revealed that the mice vaccinated with planktonic vaccine by using s/c route produced significantly higher ($p<0.05$) humoral immune response (IgG_1 and IgG) than the biofilm vaccine delivered using both s/c and i/mam routes. However, no detectable level of IgG_{2a} was found in mice vaccinated using both s/c and i/mam routes for the planktonic vaccine.

In contrast, the both the groups of mice vaccinated by s/c and i/mam routes produced detectable levels of IgG_{2a} in sera samples. The detection of IFN-γ level in the spelnocyte supernatant revealed that mice vaccinated with biofilm vaccine using i/mam route produced significantly higher levels than the planktonic vaccine using both s/c and i/mam routes. Observation of clinical symptoms up to 5 days of post challenge showed interesting results. All the four groups of control mice vaccinated by PBS demonstrated grade score of +++ with all the signs of mastitis including redness, swelling, exudate and discolouration of mammary gland. Mice vaccinated by planktonic vaccine using s/c and i/mam routes and biofilm vaccine using s/c route showed grade score ++ clinical symptoms. However, the mice vaccinated with biofilm vaccine using i/mam did not show any visible signs of mastitis on the first day of postchallenge and a grade score of + clinical symptoms appeared on second day postchallenge which lasted till the 5^{th} day of observation. The bacterial load study of mammary glands collected from mice on the 5^{th} day of postchallenge showed that the mice vaccinated with the biofilm vaccine using both s/c and i/mam routes had significantly less number of log average of *S. aureus* CFU in the mammary glands ($p<0.05$) than the mammary glands of mice vaccinated with planktonic vaccine using both s/c and i/mam routes and the control groups of mice. However, mice vaccinated with planktonic vaccine using s/c route showed significant difference in the total *S. aureus* load in mammary gland than the control group of mice. But no significant difference could be observed between total *S. aureus* load in mammary gland in mice vaccinated by planktonic or biofilm vaccine regardless of the route of immunisation. Histopathology study of the mammary tissue supported the finding of bacterial load. The histopathological grading of the mammary tissue lesions in all the groups except biofilm (i/mam) group was observed to be 3. In these groups, well preserved mammary tissue showed the evidence of intramammary abscess with central liquefactive necrosis that was walled off by a fibrinous capsule (Figure 8.8). Numerous gram positive cocci within liquefactive necrosis and lumina of mammary gland were observed. However, mice vaccinated with biofilm i/mam route showed less than grade 2 (average of 1.67) histopathological changes in mammary tissue with minimal to moderate inflammatory response and sporadic or no presence of bacteria in mammary tissue. Though there was reduction in the severity of tissue damage in the mammary glands in mice immunised with the killed whole cell biofilm vaccine by i/mam route,

complete prevention of tissue damage could not be observed against a heavy challenge dose sufficient to induce clinical mastitis.

In summary, it can be concluded that a vaccine comprising of killed whole cell of *S. aureus* residing in biofilm and delivered via i/mam route induces protective immune response potentially due to the induction of cell-mediated immunity against mastitis caused by *S. aureus* and reduced the colonization of *S. aureus* in mammary gland, severity of clinical symptoms and tissue damage in mammary gland was lessened. The planktonic vaccine despite producing significantly higher humoral immune response was not protective as judged by the level of tissue damage, clinical severity and colonization of *S. aureus* in mammary gland. If the challenge dose was lower as is observed in chronic mastitis, complete protection may have been observed. However, this study proved the concept underpinning the hypothesis that a biofilm vaccine will be more protective than the planktonic vaccine.

The above findings lay the foundation for future research on the identification of the novel antigens present in the extracellular matrix of biofilm and evaluation of their immunogenicity and protective potential using the mouse mastitis model. Regardless, it has become clear that immunisation by the intramammary route is the better route of immunisation than the subcutaneous route because of generation of both humoral and cell-mediated immune responses against *S. aureus*-associated bovine mastitis.

Chapter 9: Evaluation of immunogenicity and protective potential of Protein A against bovine mastitis caused by *Staphylococcus aureus* using mouse mastitis model

9.1 Introduction

The two most important immune evasion antigens of *Staphylococcus aureus* are Protein A encoded by the *spa* gene and capsular polysaccharide (Forsgren, 1970; O'Riordan and Lee, 2004). Capsular polysaccharides are poorly immunogenic and have been evaluated for their vaccine potential against *S. aureus* infections in humans as conjugate vaccines (Middleton, 2008). It has been reported to be an important MSCRAMM mediating formation of biofilm (Merino et al., 2009) although more recently it was reported that there was no association between capsular polysaccharide and biofilm formation (Babra et al., 2013). The N-terminal region of *spa* contains immunoglobulin binding domains (Sjodahl, 1977) which can bind to the Fcγ and Fab portions of IgG and IgM, respectively leading to suppression of immune response including both innate and adaptive immunity (Kim et al., 2010). Besides, *spa* is capable of inhibiting opsonisation of *S. aureus* hindering complement-binding sites of immunoglobulins resulting prevention of activation of available alternative complement pathway (Spika et al., 1981). In addition, the immunoglobulin binding domains of Protein A can further interact with the von Willebrande factor (vWF) assisting adherence of *S. aureus* cells onto vascular endothelial cells (Kim et al., 2012). Besides its intense effects on innate immune system, Protein A suppresses adaptive immune system by interacting with VH^3- type of IgM which is expressed in around 30% of B lymphocytes of human origin (Kim et al., 2012). This interaction is responsible for down regulation of B cell receptors (Kim et al., 2012) resulting apoptosis of the B lymphocytes (Goodyear et al., 2004). Due to the immune evasion nature of Protein A, it is considered one of important virulence determinants of *S. aureus*. Various studies performed in murine model have demonstrated the importance of *spa* in the virulence of *S. aureus* associated infections (Kim et al., 2010; Kim et al., 2012).

In one study, comparison between a *S. aureus* mutant strain devoid of *spa* gene and a wild type *S. aureus* was studied in murine bacteraemia model (Kim et al., 2010). Mice were infected via intravenous route and the rate of mortality recorded. There

was significant lower mortality in mice infected with the mutant strain of *S. aureus* than the wild type. This study has demonstrated the importance of Protein A as an important vaccine candidate to be considered while formulating a vaccine against *S. aureus*-associated infections. Mice immunised with a modified non-toxic form of protein A spA_{KKAA} was shown to protect against renal infections with a reduction in bacterial load in renal tissue samples (Kim et al., 2010). spA_{KKAA} was also found to boost humoral immune response by producing antibodies to neutralize Fcγ/Fab binding characteristics of *spa* (Kim et al., 2012). However, contrast to these studies, *spa* was not found to be a contributor of virulence of *S. aureus* when studied in mouse mastitis and rabbit keratitis models (Jonsson et al., 1985; Callegan et al., 1994).

In our previous study to investigate the prevalence of Protein A in 154 strains of *S. aureus* isolated from bovine mastitis cases in Australia, it was revealed that a total of 135 (87.7%) of *S. aureus* strains were found to posess the *spa* gene (Refer Chapter 4 Section 4.3). Based on this information, Protein A was chosen as one of the vaccine candidates to study its immunogenicity and protective potential against bovine mastitis using the mouse mastitis model.

9.2 Material and Methods

9.2.1 Detection of immune response generated by protein A using mouse mastitis model

9.2.1.1. Animal ethics approval

A total of 24 numbers of 3 days pregnant Balb/c mice (6 mice per group for four different groups including control) were obtained from the Animal Resources Centre, Perth, Western Australia and used for the study. All animal experiments were carried out with the approval of Curtin University's Animal Ethics Committee (Approval No: AEC_2013_24) ensuring compliance with the Western Australian Animal Welfare Act, 2002.

9.2.1.2 Immunization procedure

The experimental mice (n=24) were divided into four groups, each group comprising 6 mice (Table 9.1). One group of mice was immunised with Protein A by s/c route

whereas the second group was vaccinated by i/mam route. The remaining two groups were control mice for s/c or i/mam routes. 25µg of proteinA (Sigma Aldrich, Australia) was dissolved 50µl PBS. It was mixed with 50µl of Imject Alum (Thermo Fisher Scientific) and a total of 100 µl was administered to the mice on days 0, 7 and 14 day, respectively using s/c and i/mam routes. For i/mam inoculation, mice were anaesthetised with intraperitoneal (i.p.) dose of 100 mg ketamine kg−1 and 10 mg xylazine kg−1, put on their back under a binocular and the teats and the surrounding area were disinfected with 70% ethanol. The hind teat, numbered R5, was held with fine forceps and the duct orifice was located. A volume of 0.1 ml of proteinA with adjuvant was injected through the orifice using 31 gauge blunt needles. The mice were monitored twice a day. Blood and spleen were sampled on 28^{th} day for collection of sera for antibody isotyping and cytokine analysis, respectively.

9.2.1.3 Detection of antigen-specific antibody isotypes in serum

Estimation of IgG, IgG_1 and IgG_{2a} anti-*S.aureus* specific antibody isotypes was carried out on both immunised and control mice sera samples. The antibody titres were determined by using an indirect ELISA (Chapter 5, Section 5.2.3.1).

9.2.1.4 Estimation of interferon gamma (IFN-γ) in splenocyte supernatants

Estimation of IFN-γ, as an indirect indicator of cell mediated immunity (CMI) was carried out for the vaccinated and control groups of mice using ab46081 IFN gamma Mouse ELISA kit (Abcam®) following manufacturer's instructions. Methods for the stimulation of splenocytes cultured *in vitro* and collection of splenocyte supernatants have been detailed in Chapter 2 Section 2.5.

9.2.2 Determination of protective potential of Protein A using the mouse mastitis model

9.2.2.1 *S. aureus* strain

A strong biofilm former BOAISRF *S. aureus* 51 with capsular polysaccharide type 8 and carrying both *ica*A and *ica*D loci was chosen to be used in the challenge experiment (Details of phenotypic characteristics provided in chapter 8, Section 8.2.1.1). This strain was selected from a collection of 154 bovine mastitis-associated *S. aureus* strains obtained from Victoria and Queensland, Australia.

9.2.2.2 Animal ethics approval

A total of 24 numbers of 3 days pregnant Balb/c mice (6 mice per group for four different groups including control) were purchased from the Animal Resources Centre, Perth, Western Australia and used for the study. All animal experiments were carried out with the approval of Curtin University's Animal Ethics Committee (Approval No: AEC_2013_24) ensuring compliance with the Western Australian Animal Welfare Act, 2002.

9.2.2.3 Preparation of bacterial inocula for challenge

BOAISRF *S. aureus* 51 was grown on MH agar plates at 37°C for 18h. The pure colonies were washed from the plates using 20 ml of isotonic saline and suspended in isotonic saline to give a final viable bacterial count of 4 x 10^{11} ml^{-1} (Anderson and Chandler, 1975).

9.2.2.4 Challenge experiment

A total of 24 mice vaccinated with 3 doses of Protein A were challenged on 3-5 days of lactation (Table 9.1). Both L5 and R5 mammary glands of 24 lactating mice from each treatment group were injected with 50 µl (CFU 2x10^{10}) of CP8 positive, strong biofilm former strain, BOAISRF *S. aureus* 51 strain. Mice were challenged via intra-mammary route using thirty one gauge needles. The mice were observed twice daily for morbidity and mortality until 5 days post-challenge. Percent protection against mouse mastitis was recorded and surviving mice euthanized after sampling blood and mammary glands.

Table 9.1: Experimental groups of mice used in the immunogenicity and protective potential trials of Protein A vaccines (n=48)

Sl. No	Group	Number of mice for immunogenicity trial	Number of mice for protective potential trial
1	Protein A s/c route	6	6
2	Protein A i/mam route	6	6
3	PBS control for s/c route	6	6
4	PBS control for i/mam route	6	6

9.2.2.5 Clinical observation

The mammary glands of mice were observed for clinical signs of mastitis including redness, swelling and discolouration of mammary gland and extrusion of exudate with or without squeezing of the mammary gland (Chapter 6, Section 6.2.5.1).

9.2.2.6 Bacterial load study of mammary glands

The L5 mammary glands from control and test mice were collected and subjected to bacterial load study (Chapter 6, Section 6.2.5.2.1).

9.2.2.7 Histological observation

R5 mammary glands were collected from control and test mice were collected for histopathological examination (Chapter 6, Section 6.2.5.3.1).

9.2.2.8 Statistical analysis

To compare the antigen specific antibody levels, Interferon gamma (IFN-γ) levels and Log average number of bacteria (CFU) recovered from mammary glands between groups of mice immunized with planktonic and biofilm *S. aureus* vaccines, student's t-test was performed. Statistical significance was set at $p < 0.05$.

9.3 Results

9.3.1 Detection of antigen-specific antibody in sera samples of mice vaccinated with ProteinA

The titre of IgG, IgG_1 and IgG_{2a} was determined by using indirect ELISA. The overall titre of IgG, IgG_1 and IgG_{2a} in mice vaccinated by s/c route was 531.67 ±146.21, 306.67±58.35 and 116.67±17.45, respectively (Figure 9.1A) and 242.5±9.29, 46.67±6.67 and 126.67±4.22, respectively for the mice vaccinated by i/mam route (Figure 9.1B).

9.3.2 Interferon gamma (IFN-γ) levels in splenocyte supernatants

Splenocytes of mice vaccinated with Protein A by both s/c and i/mam routes produced low levels of IFN-γ when stimulated with Protein A. However, splenocytes stimulated with ConA produced very how level of IFN-γ (5700 pg/ml). In s/c and

i/mam vaccination groups, IFN-γ levels in Splenocytes stimulated with Protein A produced 159±9.18 pg/ml and 186.34±20.09 pg/ml, respectively (Figure 9.2).

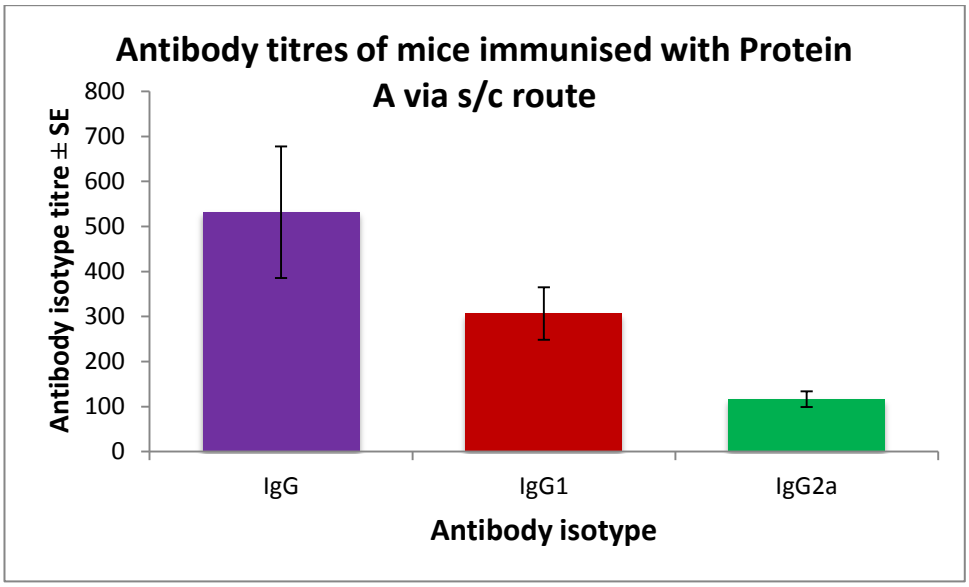


Figure 9.1A: Antibody isotypes of mice vaccinated with Protein A by s/c route

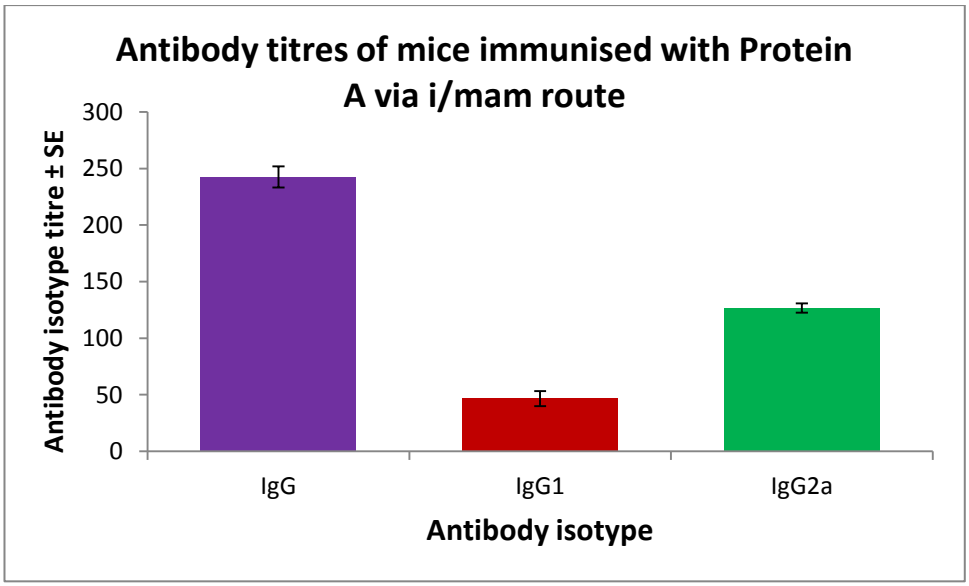


Figure 9.1B: Antibody isotypes of mice vaccinated with Protein A by i/mam route

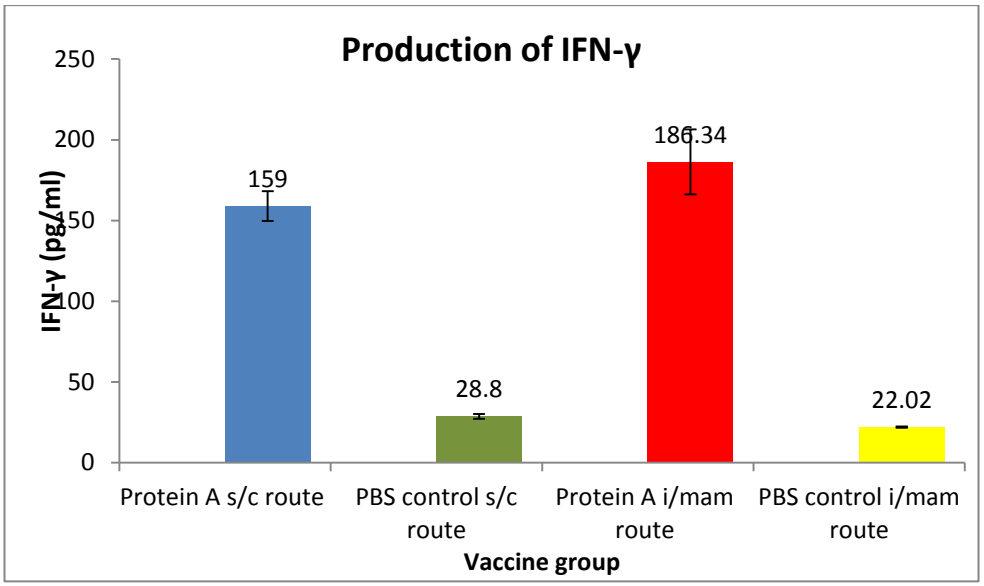


Figure 9.2: Concentration of IFN-γ produced by splenocytes of Protein A stimulated mice immunized by Protein A using different routes.

9.3.3 Clinical observation of mammary glands of challenged mice

The control group showed signs of mastitis in the inoculated glands. All of the test mice showed varying degrees of changes in the gross clinical appearance of mammary glands (Table 9.2).

Table 9.2: Clinical signs observed in different groups of mice immunized with Protein A (Observations up to 5 days post challenge)

Level of clinical signs* observed in test mice injected with Protein A					
Vaccination	**Day1**	**Day2**	**Day3**	**Day4**	**Day5**
ProteinA (s/c route)	++	+++	+++	+++	+++
ProteinA (i/mam route)	++	++	++	+++	+++
PBS (s/c route)	++	+++	+++	+++	+++
PBS (i/mam route)	++	+++	+++	+++	+++

*Clinical features include redness, swelling, and discolouration of mammary gland, exudate, morbidity and mortality. Grade scores compare observed features to the most severe changes: 0 - no macroscopic changes, + low grade, ++ medium grade, +++ severe grade.

9.3.4 Bacterial loads in the mammary glands of challenged mice

The bacterial load of mammary gland of mice vaccinated with Protein A by i/mam route was 7.33±0.29 CFU compared to vaccination of mice by the s/c route with significantly higher ($p<0.05$) bacterial load of 8.18±0.14 CFU (Table 9.3). In paired *t* test between Protein A vaccine (s/c and i/mam routes) with control PBS immunized mice (s/c and i/mam routes) demonstrated significant difference (< 0.05) in bacterial load of mammary glands between Protien A (i/mam route) vaccinated and control mice.

9.3.5 Histopathological observation of mammary glands of challenged mice

Histopathological grade observed in the mammary tissue are shown in Table 9.2 and Figures 9.3-9.10. There was no significant difference ($p>0.05$) detected in histopathological grades of mammary tissue lesions between Protien A vaccinated and control mice.

Table 9.3: Detection of bacterial load and histopathological changes in the mammary glands of mice immunized with Protein A

Group	Vaccine group	Total number of mammary glands investigated	Log average number of bacteria (CFU) recovered from mammary glands ± SE	Grades for histopathological changes					
				M1	M2	M3	M4	M5	M6
1	ProteinA s/c route	6	8.18±0.14	3	3	3	3	3	2
2	ProteinA i/mam route	6	7.33±0.29	3	3	3	3	2	3
3	PBS control s/c route	6	9.04±0.06	3	3	3	3	3	3
4	PBS control i/mam route	6	9.05±0.04	3	3	3	3	3	3

M*= Mammary gland

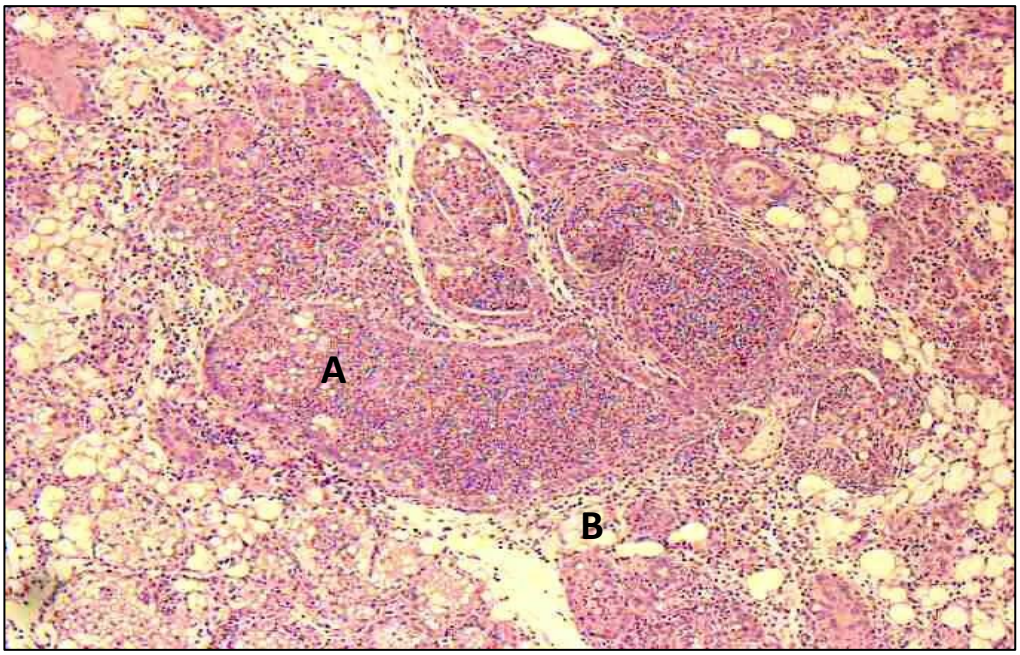


Figure 9.3: Mammary tissue specimen from lactating mouse immunized with Protein A vaccine (s/c route) and challenged using strong biofilm forming *S. aureus* 51. Marked inflammatory cell infiltration within intralobular (A) and perilobular tissue (B). Level 3 category inflammation. H&E x 100mag.

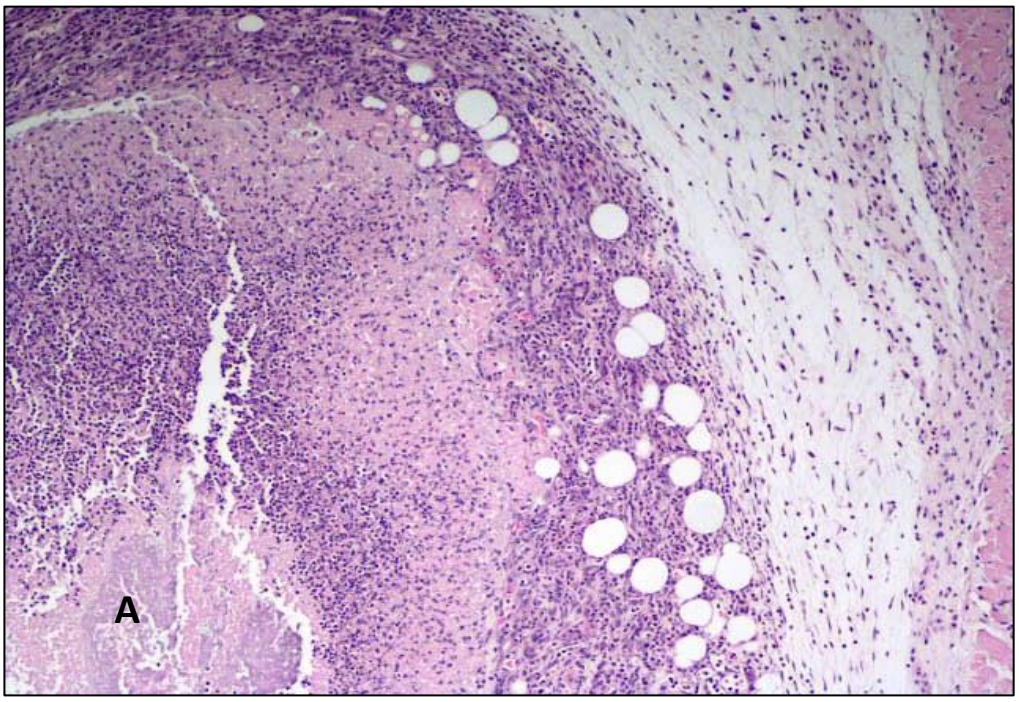


Figure 9.4: Mammary tissue specimen from lactating mouse immunized by Protein A vaccine (i/mam route) and challenged using strong biofilm forming *S. aureus* 51. Abscess in mammary tissue (A). Level 3 category inflammation. H&E x 100mag

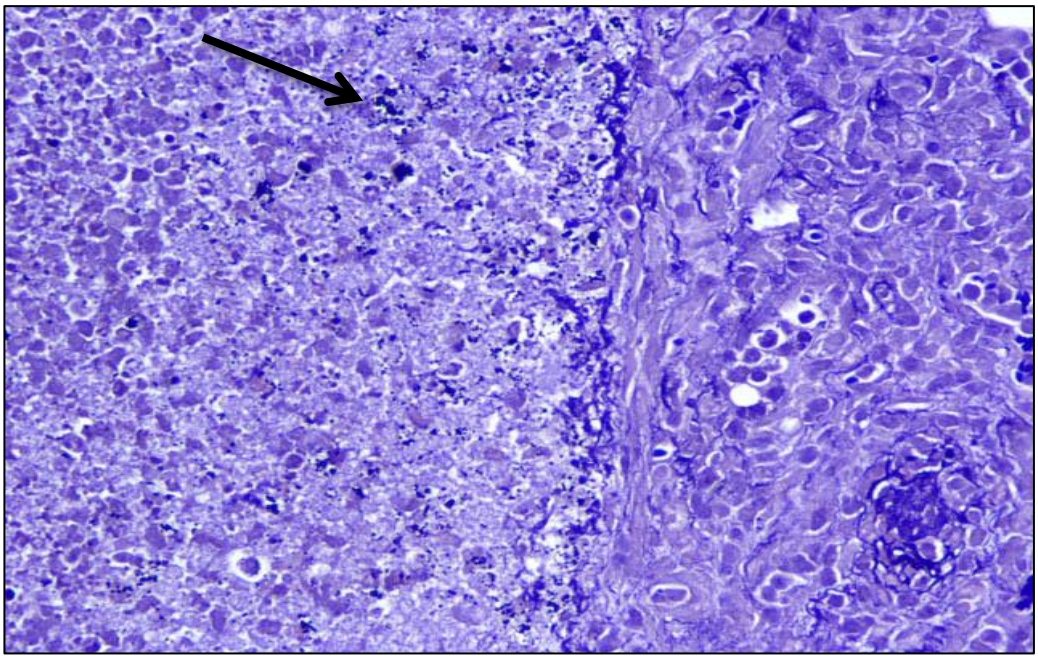
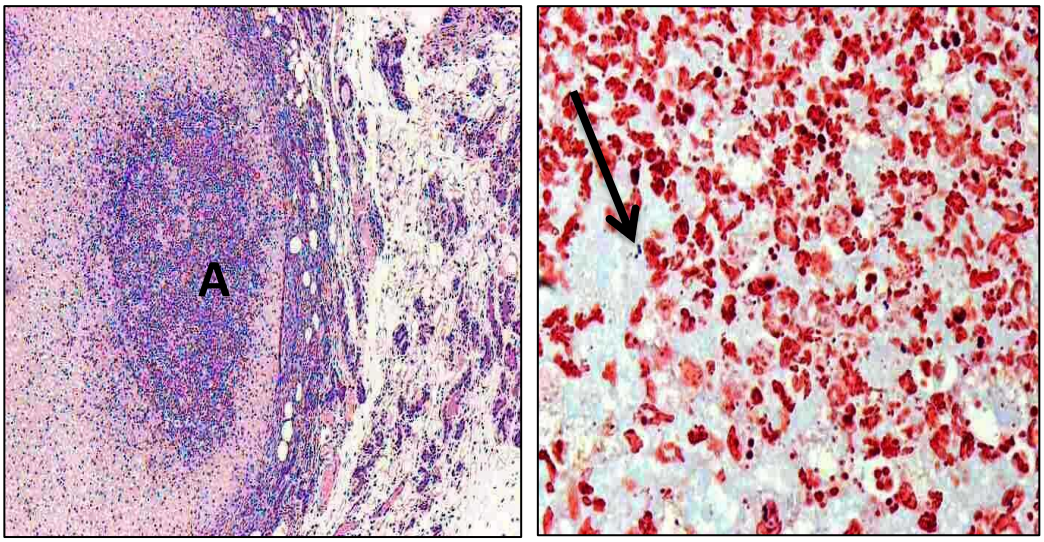


Figure 9.5: Mammary tissue specimen from lactating mouse immunized by Protein A vaccine (i/mam route) and challenged using strong biofilm forming *S. aureus* 51. Gram positive bacteria and associated inflammatory cell infiltrate (Arrow). Gram Twort x 400mag.

Figure 9.6: Mammary tissue specimen from lactating mouse injected with PBS and challenged using strong biofilm forming *S. aureus* 51. Abscess in mammary tissue with tissue necrosis (A). Level 3 category inflammation. H&E x 40mag.



9.7 (a) 9.7 (b)

Figure 9.7: Mammary tissue specimen from lactating mouse injected with PBS and challenged using strong biofilm forming *S. aureus* 51(from figure 9.6). 9.7(a) Inflammatory abscess shows inflammatory exudate in fibrinous capsule (A).). Level 3 category inflammation. H&E x 100mag. 9.7(b) Gram positive bacteria (Arrow) in inflammatory material Gram Twort x1000mag.

9.4 Discussion

The study of immune response generated by Protein A in the mouse mastitis model showed that mice vaccinated by subcutaneous and intra-mammary routes produced all the three antibody isotypes (IgG, IgG1 and IgG2a) in sera samples. However, the titre of all the three antibodies were significantly low, IgG2a being the lowest than those estimated for the killed whole cell biofilm vaccine (Refer Chapter 8 Section 8.3.1.2). Intra-mammary route administration of protein A induced comparatively better IgG2a than the subcutaneous route. The detection of IFN-γ levels as indirect indicator of cell-mediated immunity in splenocyte supernatants in both subcutaneous and intra-mammary routes of vaccinated mice indicated production of lower levels of IFN-γ and intra-mammary route of vaccination producing slightly higher level of IFN-γ.

Upon challenge by the CP8 positive, biofilm forming and *S. aureus* strain, the mortality rate and clinical symptoms developed in mice were recorded for up to 5 days post-challenge. There was no mortality of mice up to 5 days post-challenge. However, varied levels of clinical symptoms were observed. There was no difference

between the levels of clinical symptoms between PBS control and Protein A group of mice vaccinated by subcutaneous route. The group of mice which were vaccinated with Protein A by intra-mammary route showed medium level of clinical symptoms up to 3 days, though on 4^{th} day onwards there was no difference between the levels of clinical severity among all the groups of mice.

The bacterial loads of the mammary glands of all the four groups of mice showed high bacterial load ranging from log average number (CFU) 7.33±0.29 to 9.05±0.04. However, the mammary glands of mice vaccinated by intra-mammary route had significantly lower numbers ($p<0.05$) of bacteria than the other groups.

Histopathological analysis of the tissue sections did not project any difference between the mice immunized with Protein A using either subcutaneous or intra-mammary routes or the control mice immunized using PBS. All the four groups of test and control mice showed almost identical picture of mammary tissue damage.

It can be concluded that mice immunized with Protein A using subcutaneous or intramammary routes yielded antibody and cell-mediated immune responses that were significantly lower than those observed in mice vaccinated with the whole cell biofilm vaccine and failed to protect the mice which were challenged with CP8 positive and strong biofilm producing *S. aureus* via i/mam route. Whether a conjugate vaccine (Protein A+CP8) will provide better immune responses and protection against challenge infection with *S. aureus* would be interesting to be determined.

Chapter 10: General discussion

10.1 Summary

Mastitis is the predominant intra-mammary infection in dairy cattle in Australia causing a huge economic loss to the dairy industry. Both clinical and subclinical bovine mastitis are responsible for reduction in milk production, deterioration of quality of milk and milk products, increased amount of health care expenditure, financial loss due to culling of sick animals and even mortality of cow. Despite application of various management practices and antibiotic therapy, mastitis is still an unresolved battle in dairy industry worldwide including Australia.

S. aureus is the prominent causative contagious agent of bovine mastitis. During 1960 to 1990, the predominant causative agent of bovine mastitis in Australia was *S. aureus* and *S. agalactiae.* However, in the past 20 years, the environmental pathogen such as *S. uberis* has become the predominant mastitis pathogen in Australia. While the incidence of mastitis due to environmental pathogens can be reduced by improvement in management practises such as cleaning of udder before and after milking, pre and post-milking teat dipping, adequate nutrition with selenium and vitamin E supplementation, use of teat sealant in dry cows, culling etc. The only effective treatment regimen against mastitis caused by *S. aureus* is the use of antimicrobial agents to combat intra-mammary infection. But ability of this pathogen to develop antibiotic resistance within a short span of time has made conservative treatment approach unsuccessful. Besides, those cases where treatment is successful to control mastitis, the somatic cell count remains higher or reduced and starts increasing after a short period (Melchior et al., 2006). Success of antibiotic therapy depends on various factors. These factors include serum protein binding, solubility of lipid, pK_a value of used antimicrobial agent (Melchior et al., 2006), composition of antibiotic preparation (Du Preez, 2000), level of tissue damage in mammary gland (Shoshani et al., 2000). Due to these factors the cure rate of mastitis caused by bacterial pathogen ranges from 0-80% in general and only 0-52% for lactating animals (Melchior et al., 2006).

Since 1990 there has been an increasing emphasis on another important aspect of *S. aureus*, the biofilm forming potential. Biofilm producing *S. aureus* can attach more

effectively to the epithelial lining of mammary gland to develop intra-mammary infection (Baselga et al., 1993). In fact, *S. aureus* isolated from mammary gland are more likely to form biofilm than *S. aureus* isolated from external sources including milking machine (Fox et al., 2005). Besides, bacteria growing in biofilm demonstrate 10-1000 times increased resistance to antimicrobial therapy than its planktonic form of growth (Olson et al., 2002). This resistance to various antimicrobial agents may be due to delayed penetration of antimicrobial agents crossing the barrier of slimy biofilm matrix, modification in the growth rate of pathogen residing in biofilm and certain physiological and genotypic changes in pathogen residing in biofilm (Melchior et al., 2006).

S. aureus produces a wide range of virulence factors ranging from capsular and surface polysaccharides, MSCRAMMS, toxins, superantigens, enzymes etc which make the *S. aureus* a versatile one among all the other bacterial pathogens associated with intramammary infection. Moreover, the mechanism of pathogenicity and protective immune mechanism against *S. aureus* are still not clear. In addition, *S. aureus* is capable of neutralizing the host immune response more effectively than any other bacterial pathogen (Lowy, 1998). All these factors have collectively contributed to the difficulty to develop a potential vaccine against *S. aureus*-associated infections. Numerous clinical trials have been completed in both human and animals and different antigens have been targeted to stimulate immune responses which have all ended essentially in failure (Leitner *et al*, 2003b; Leitner et al., 2003c; Middleton, 2008).

Various reasons can be hypothesized for the failure of developing an effective vaccine against *S. aureus* infections. These reasons include (a) failure to choose the right combination of antigens as vaccine candidates or (b) a reduction in opsonic killing capacity due to competitive inhibition of bacterial activity of antibodies directed against different antigens (Skurnik et al., 2010). Moreover, use of animal models although informative, cannot be equated to the actual host of the *S. aureus* associated-infection i.e the ruminant species.

Taking into consideration the previous efforts of vaccine development and their failures, this project was undertaken using an entirely different approach. We decided

to evaluate the immunogenicity and protective potential of a vaccine candidate that should potentially present as many of the MSCRAMMS including the capsular polysaccharides and PNAG, hence to use killed whole cell of *S. aureus* residing in biofilm versus killed planktonic whole cell vaccine administered by s/c versus i/mam route. It was hypothesized that mice vaccinated with bacterins from strong biofilm producing *S. aureus* will generate better immune responses and confers better protection against mastitis following challenge with a biofilm producing *S. aureus* than a planktonic vaccine. The genes expressed by *S. aureus* in biofilm and host immune response against *S. aureus* in biofilm has been reported to be different from that induced by *S. aureus* cells in the planktonic form (Prignent-Combaret et al., 1999). To arrive at our ultimate goal of formulating a suitable biofilm based vaccine against *S. aureus*-associated bovine mastitis in Australia, investigations on the biofilm forming potential of various clinical *S. aureus* isolates of bovine mastitis origin in Australia and prevalence of the detectable virulence factors in these isolates were carried out. Furthermore, a non-invasive method for production of mastitis in mice was developed to enable evaluation of the immunogenicity and protective potential of vaccine candidates.

Virulence of *Staphylococcus* species in chronic and local infections like mastitis may be largely dependent on the bacterium's ability to form biofilms (Mah and Toole, 2001). Our study has evaluated the biofilm forming potential of 154 clinical isolates of *S. aureus* isolated from bovine mastitis in Australia using the Tissue Culture Plate (TCP) and the Congo Red Agar (CRA) methods. TCP method was found to be a better indicator test of biofilm-forming potential of *S. aureus.* A substantial number of biofilm-producing isolates was missed when the CRA method was used alone. TCP method could determine biofilm forming ability of 100% of the strains, whereas CRA method could detect only in 31.17% of the biofilm producing strains.

Majority of the *S. aureus* strains used in this study were sensitive to the 12 different antibiotics used to perform antimicrobial susceptibility test. A total of 29 (18.83%) *S. aureus* strains were found to be resistant against penicillin which is followed by 3.25% and 2.6% resistance against ciprofloxacin and erythromycin, respectively. Twenty eight *S. aureus* strains which were initially susceptible in planktonic form to 12 different antibiotics were selected to confirm the development of resistance to

different antibiotics in the biofilm to many antibiotics developed but it also persisted for up to 4 weeks when grown as planktonic culture before reverting back to antibiotic susceptibility. The study suggests that (a) biofilm formation assays should be carried out using more than one method, and (b) antibiotic sensitivity tests on clinical samples should include both the planktonic and biofilm forming cultures, with a view to improving the therapeutic outcomes in the treatment of bovine mastitis caused by *S aureus*, and potentially other biofilm-producing pathogens.

In the present study, >70% isolates producing biofilm were found to carry both *ica*A and *ica*D genes but the presence of *ica* locus was did not correlate with biofilm formation by the isolates. The present study also could not find a correlation between biofilm formation and presence of *bap* gene and *agr* types in *S. aureus*. The investigation also demonstrated lack of correlation between encapsulation and biofilm formation since non-encapsulated clinical *S. aureus* isolates were also found to form biofilms confirming results reported previously (Babra et al., 2013).

Another study was conducted to determine the distribution of virulence factor-encoding genes in the 154 clinical *S. aureus* isolates from bovine mastitis in Australia. By using serological methods and conventional PCR developed in this investigation, it was revealed that *clfA* (91.56%), *clfB* (92.86%), *spa* (87.7%), *fnbpA* (54.5%), *isdA* (98.1%), *isdB* (100%), *sdrD* (98.1%), *sdrE* (95.5%) genes were the predominant MSCRAMMS-encoding genes in *S. aureus* of clinical bovine mastitis origin. The predominant enterotoxin encoding genes were *seh* (32.5%), *sec* (23.4%), *seg* (17.5%) and *sei* (13%) in bovine *S. aureus* isolates, with *sea*, *seb*, *sed*, *see*, *sej* being detected in very low numbers ranging from 0.65-2.6%. However, detection of virulence factors in *S. aureus* isolates has been limited to the human origin in Australia. No information on the prevalence of virulence factors including MSCRAMMS, superantigens and cytotoxins associated with Australian bovine mastitis *S. aureus* isolates are available in the literature. This study suggests considering predominant MSCRAMMS such as ClfA, ClfB, ProteinA, isdA, isdB, SdrD, SdrE and FnBpA as potential vaccine candidates against bovine mastitis caused by *S. aureus* in Australia. Given the presence of the genes encoding α and β toxin in majority of the *S. aureus* isolates (94.16 % α toxin and 83.12 % β toxin) and their role in biofilm formation it is also important to consider these as important

potential vaccine candidates for the development of biofilm antigen based vaccine against bovine mastitis.

S. aureus, a predominant contagious pathogen causing bovine mastitis, expresses capsular polysaccharides (CPs) which are important virulence determinant (Lee et al., 1987). CPs contribute to the virulence of *S. aureus* by resisting phagocytosis and thus allowing persistence of the pathogen in tissues of infected host and promoting formation of abscess (O'Riordan and Lee, 2004). Based on the recent classification suggested by Lee (2002), *S. aureus* CPs can be classified into four different types: CP1, CP2, CP5 and CP8. CP 1and CP2 and heavily capsulated and produces mucoid colonies on solid medium (Karakawa and Vann, 1982). However, CP 5 and CP8 *S. aureus* do not produce mucoid colonies and are the most prevalent CP types in clinical isolates from human as well as cows (Verdier et al, 2007; Gogoi-Tiwari et al., 2014 unpublished data). CP5 and CP8 *S. aureus* are referred as microencapsulated strains due to their ability to produce limited capsule in broth culture. The *S. aureus* isolates carrying none of the four CP types are classified as non typeable and majority of these strains were reported to possess a unique surface polysaccharide antigen 336 (Guidry et al., 1998), which are reported to be composed of polyribitol-phosphate-*N*-acetylglucosamine, a component of cell wall teichoic acid (Verdier et al., 2007).

There is no data available in the literature on the prevalence of capsular polysaccharide types in *S. aureus* isolates of bovine mastitis origin in Australia. Gaining this knowledge is important for development and evaluation of vaccines as a strategy for prevention of bovine mastitis. Our study has determined the prevalence of the different capsular polysaccharide (CP) and the major surface-associated non-CP antigen 336 (SP-336) types among 154 *S. aureus* isolated from bovine mastitis cases in Australia. Mouse antisera raised against CP types (CP1, CP2, CP5, and CP8) or SP-336 were used in slide agglutination tests and compared to detection of *cap*1, *cap*5 and *cap*8 gene fragments by PCR. Serological studies revealed the presence of CP2, CP5, CP8 and SP-336 in 9.09, 23.38, 31.82, and 5.84 percent of the Australian *S. aureus* isolates, respectively. By PCR, CP1, CP5 and CP8 accounted for 0, 26.62 and 32.47 percent of the *S. aureus* isolates of mastitis origin in Australia. Both PCR and serological study have demonstrated that CP5 and CP8 were the predominant

capsular types in Australia. The study has also demonstrated a strong correlation between both the methods of typing used for CP1, CP5, CP8 and non-typeable *S. aureus*. Capsular staining method has confirmed the result of serological detection of capsule in *S. aureus* isolates. Besides, out of 55 non typeable strains detected in serological method, 13 *S. aureus* strains were found to be positive for capsules in negative staining method. This is an important finding of this study which suggests that there may be more than the 5 (4 CP types and 1 SP type) recognised types of CP and SP in *S. aureus* as proposed originally by Karakawa and Vann (1982).

Variety of intra-mammary inoculation techniques have been used to produce mastitis in the mouse model system. However, However, these techniques have included inoculation of bacterial suspension by dropping it on the tip of mammary teat and allowing absorption (Anderson, 1979), inoculation of bacterial pathogen by passing a micropipette of less than 75µm diameter through teat canal (Lee et al., 2003), injection of inoculum by using a 30 gauge needle after incising the near end of teat (Brouillette and Malouin, 2005) and surgical exposure of mammary gland followed by injection of bacterial suspension into primary duct by using a 30 gauge needle (Nguyen et al., 2000). These methods have one or the other disadvantages such as trauma and rupture of the mammary teat leading to failure of the techniques or chance of secondary bacterial contamination at the inoculation site. While standardizing the mouse mastitis model, one of the objectives was to improvise the method of inoculating mammary gland without traumatising the teat lactiferous duct or the tissue encasing the gland with the intention of mimicking the natural mode of infection. Besides, the study was undertaken to determine the impact of the strength of biofilm formation on the extent of damage to the mammary tissue caused by encapsulated *S. aureus* isolates using the non-invasive mouse mastitis model system developed in this project.

Four strains of *S. aureus* of the same capsular phenotype (CP8) but variable in the strength of biofilm formation were used in this study. The delivery of the infectious dose of *S. aureus*, performed without invasive scraping of the teat surface, was carried out directly through the teat lactiferous duct using a syringe fitted with a blunt ended 31-gauge hypodermic needle. Histopathological analysis showed that mice infected with encapsulated (CP8) strong biofilm forming *S. aureus* with *ica*A

and *ica*D genes produced marked acute mastitis lesions characterised by a profuse inflammatory infiltration with evidence of necrosis in the mammary glands (Chapter 6 Figure 6.1d). The damage was significantly less severe in tissue sections from mice injected with the weak biofilm-forming *S. aureus* in absence of icaA and icaD genes (Chapter 7 Figure 7.1). A significant increase in the inflammatory cytokines IL-1β and TNF-α level determined by flow cytometry was found to correlate with the extent of the tissue damage suggesting a potential role of biofilm formation and inflammatory cytokines in the pathology of bovine mastitis. This study has demonstrated that mastitis can be successfully produced in the mouse model by direct inoculation of *S. aureus* into the mammary gland via the mammary duct without scrapping off the mammary gland surface epithelium or glands. The bacteriological and histopathological study revealed proliferation of the bacteria as well as inflammatory infiltrates in mammary glands and not in the blood or liver or spleen ruling out sepsis. Use of a direct delivery inoculum in a mouse mastitis model may minimise the chance of secondary bacterial infection with skin associated opportunistic pathogens such as *Pasteurella pneumotropica*, *S. aureus* and *Corynebacterium bovis* (Baker, 1998), which can occur in the case of invasive mouse mastitis model where the tip of the mammary gland is scrapped or incised (Brouillette and Malouin, 2005; Brouillette et al., 2005).

Given the finding that strength of biofilm formation by encapsulated *S. aureus* correlated with extent of tissue damage to the mammary gland with no observed mortality. It was important to determine if the same would hold true for strong versus weak biofilm forming non-capsulated i.e non-typeable *S. aureus*. However, surprising and unexpected finding was made. Mice infected with two different non-typeable strains strong biofilm forming BOAISRF *S. aur* 83 versus weak biofilm forming BOAISRF *S. aur* 87 showed surprising results. All the mice inoculated by BOAISRF *S. aureus* 83 and 87 died within 24 and 30 h of post infection, respectively. However, the histopathological analysis of mammary tissue damage revealed minimal inflammatory infiltrates in the mammary gland. This was unexpected because of the absence of one major immune evading capsular antigen. Flow cytometry analysis of sera samples collected from mice immediately before death showed higher levels of IL-1β, IL-6, IL-10, IL-17A, IFN-γ and TNF-α than the sera of control mice. The highly elevated level of various cytokines can lead to

cytokine storm, a fatal immune response which may result in sudden death (Osterholm, 2005) as human patients died due to cytokine storm despite higher levels of anti-inflammatory cytokine, IL-10 but the presence of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α in serum samples (Horst, 2002). The pro-inflammatory cytokines IL-1β, IL-6 and TNF-α produced by BOAIRSF *S. aureus* 83 was significantly higher ($p < 0.05$) than those induced by and BOAIRF *S. aureus* 87 infection. The level of IL-6 in serum sample of mice injected with the BOAISRF *S. aureus* 83 was as high as 15479.9±532 pg/mL. The reason for the observed quick mortality in mice infected with NT *S. aureus* strains used in this project was not investigated due to time constraints. However, our hypothesis is that the presence of capsular polysaccharides attenuates the release of the pore forming toxins such alpha and beta toxins and superantigenic exotoxins such the enterotoxins *in vivo*. Since the NT strains used in this project were non-encapsulated, significantly more toxins would have been released *in vivo*, hence the observed quick mortality. Validation of this hypothesis warrants further investigations.

Based on this observation, anti-IL-6 and IL-6Rβ antibodies were selected for use in the immunotherapy experiment. The study revealed that mortality was delayed for 12 h 30 min and 13 h 15 min in the mice treatment with anti-IL-6 and anti-IL-6R antibodies, respectively. Severity of mastitis was also reduced in terms of clinical symptoms as was the bacterial load of the mammary glands. The clinical menifestations were reduced from medium to low grade and there was significant difference in the load of *S. aureus* in the mammary glands of mice treated with anti-IL-6 and IL-6Rβ antibodies versus the PBS control groups. Although not completely prevented, but delaying of mortality time provides a window for treatment with antibiotics to prevent mortality due to infection with *S. aureus*. Whether this delay is sufficient for antimicrobial therapy resulting in prevention of death needs to be determined. However, the time constraint associated with the submission of this thesis and the end date of IPRS scholarship prevented accomplishment of this objective.

An important aim of this project was to compare the immunogenicity and protective potential of planktonic versus biofilm *S. aureus* vaccine for the prevention of mastitis using mouse as a model system. It was discovered that mice immunised with

planktonic vaccine by the s/c route induced high levels of the antibody IgG1 but lower levels of IgG2a, and the cytokine IFN-γ, indirect indicators of the induction of CMI. The immunogenicity and protective potential of the planktonic *S. aureus* vaccine delivered by the intra-mammary route, although yielded slightly lower immune responses, the protective potential was similar to that obtained with the s/c route of administration. On the other hand, high levels of IgG and IgG2a antibody isotypes and IFN-γ were induced when the biofilm vaccine was administered by i/mam route. Our approach was different from the previously reported data in that the protective potential of antibodies raised by active immunisation of mice with select biofilm antigens against systemic challenge as an indicator of protective potential (Maira Litran et al., 2005; Prenafeta et al., 2010; Skurnik et al., 2010; Gil et al., 2014). In this study i/mam route was used for vaccination as well as challenge. This is the first time in the world that superiority of biofilm *S. aureus* vaccine given by i/mam route over the planktonic vaccine has been demonstrated.

Protein A, a surface-associated molecule of *S. aureus* is an important member of MSCRAMMS, is not only an immune evasion molecule of *S. aureus*, is present in over 80% of the isolates and also mediates formation of biofilm in mammary gland, it was decided to study the immunogenicity and protective potential of this protein against bovine mastitis using the non-invasive mouse mastitis model developed in this project. It was hypothesized that if the immunogenicity and protective potential of this molecule was found to be significant, a vaccine composed of Protein A conjugated with the purified CP8, the predominant capsular polysaccharide in the Australian bovine *S. aureus* isolates could be formulated for testing as a prototype vaccine candidate. Mice immunized with Protein A by the s/c or i/mam routes and challenged with biofilm forming, CP8 positive *S. aureus* strain revealed no difference between the levels of clinical symptoms between PBS control and Protein A groups of mice. The mice group which was vaccinated by Protein A by intra-mammary route showed medium level of clinical symptoms up to 3 days, though on 4thday onwards there was no difference between the levels of clinical severity among all the groups of mice. Bacterial load study and histopathological analysis of tissue damage in mammary glands of vaccinated mice did not show any difference from the control group of mice.

Mice immunized with ProteinA by subcutaneous and intra-mammary routes produced all the three antibody isotypes (IgG, IgG1 and IgG2a) in sera samples. However, the titre of all the three antibodies were low and IgG2a being the lowest. Similarly, IFN-γ levels in splenocyte supernatants of mice immunized by intra-mammary route produced slightly higher level of IFN-γ than the subcutaneous route, but overall the levels of IFN-γ in spelocyte supernatant was very low. Since Protein A failed to produce desired immune response to prevent intra-mammary infection in mice, the planned study involving development of a conjugate vaccine using CP8 (Predominant capsular type in *S. aureus* of bovine mastitis origin in Australia) and Protein A was cancelled.

This study was therefore concluded with the findings that a vaccine comprising of formalin killed whole cell of *S. aureus* residing in biofilm and delivered via i/mam route induced a significant cell mediated immune responses (IFN-γ) and protection against mastitis caused by *S. aureus* and significantly reduced the colonization of *S. aureus* in mammary gland, severity of clinical symptoms and tissue damage in mammary gland was lessened. The planktonic vaccine although produced significantly higher humoral immune response ((IgG_1 and IgG), damage to the mammary tissue and clinical severity upon challenge could not be prevented.

10.2 Conclusion

(a) Comparative study of CRA and TCP method for detection of biofilm formation by the154 strains of *S. aureus* revealed that TCP method could determine biofilm forming ability of 100% of the strains. Whereas CRA method could only detect only 31.17% of the biofilm producing strains proving superiority of TCP method over CRA method.

(b) A high percentage (over 70%) of isolates were positive for both *icaA* and *icaD* genes, with only 2 (1.3%) of the strains being positive for *bap* gene. The present study could not find any correlation between biofilm formation and presence of *ica* loci, *bap* gene or *agr* types.

(c) All the 154 *S. aureus* strains of bovine mastitis origin against 12 different antimicrobial agents revealed all (100%) strains to be sensitive to cefoxitin, an indirect indicator of methicillin sensitivity. This was confirmed using chromogenic culture media plates, Genotype-MRSA kit and conventional PCR.

(d) Twenty eight selected *S. aureus* strains were subjected to persistence of antibiotic resistance study which were initially susceptible to 12 different antibiotics. All the strains developed resistance to more than one antibiotic after formation of biofilm and the resistance persisted as long as 4 weeks.

(e) The current study highlighted that *clfA* (91.56%), *clfB* (92.86%), *spa* (87.7%), *fnbpA* (54.5%), *isdA* (98.1%), *isdB* (100%), *sdrD* (98.1%) and *sdrE* (95.5%) were the predominant microbial surface component recognizing adhesive matrix molecules (MSCRAMMS) in *S. aureus* of bovine mastitis origin.

(f) The predominant cytotoxins produced by the *S. aureus* isolates were α-toxin (94.16%), β-toxin (83.12%). The potential super antigenic enetrotoxins produced were *seh* (32.5%), *sec* (23.4%), *seg* (17.5%) and *sei* (13%), respectively. The detection of 4 strains (2.6%) of *S. aureus* positive for *pvl* gene is an important finding as *pvl* genes are not common in bovine mastitis cases.

(g) This study revealed that majority of the Australian bovine mastitis-associated *S. aureus* isolates (64.29%) expressed capsule, of which, CP8 was predominant (31.82%), followed closely by CP5 (23.38%). In addition, 5.84% (nine of 154) of the isolates were positive for SP-336 and 30% of the isolates were non-typeable. The study further demonstrated that there was strong correlation between the serological and genotyping methods for detection of CP types 1, 5 and 8, and the non-typeable *S. aureus* isolates.

(h) The strength of biofilms produced by *S. aureus* appears to contribute to the mammary tissue damage. Histopathological analysis showed that mice infected with encapsulated (CP8) strong biofilm forming *S. aureus* produced marked acute mastitis lesions showing a profuse inflammatory infiltration with evidence of necrosis in the mammary glands. The damage was significantly less severe in sections of tissue from

mice injected with the weak biofilm forming *S. aureus* CP8 strain and there appeared to be correlation between *S. aureus* load in the mammary gland and associated tissue damage.

(i) This study has demonstrated that mastitis can be reliably produced in a mouse model by direct inoculation of *S. aureus* into the mammary gland via the mammary duct without traumatising the mammary gland surface epithelium or glands. Inflammatory cytokine, TNF-α was significantly higher ($p<0.05$) in sera sample of mice inoculated with the strong biofilm forming *S. aureus* than the weak biofilm forming strain even 48 h post infection.

(j) The mammary glands of mice infected with non-typeable strain of *S. aureus* with strong biofilm forming potential had significantly higher (≤ 0.05) load of *S. aureus* in mammary glands than the mammary glands of mice infected with non-typeable non-biofilm forming *S. aureus* strain. Although there was no significant difference observed in the level of tissue damage in mammary glands of mice infected with non-typeable biofilm producing and non-biofilm producing strains, a significantly high level of inflammatory cytokines particularly IL-6 was produced resulting in the death of animals within 48 h of post inoculation.

(k) Treatment of mice with anti-IL6 and anti IL6-Rβ antibodies delayed that mortality of mice up to 13h 15 min. Severity of mastitis was also reduced in terms of clinical symptoms and bacterial load from the mammary glands of immunized mice.

(l) This study demonstrated that mice immunized with Protein A using subcutaneous and intra-mammary routes failed to show enhanced cell-mediated or humoral immune response. The vaccine failed to protect the mice which were challenged with CP8 positive and strong biofilm producing *S. aureus* via i/mam route and there was no significant difference in the level of tissue damage between immunized and control mice raising a question on its usefulness as a carrier protein in conjugate vaccine formulations in the future.

(m) A vaccine comprising of killed whole cell of *S. aureus* residing in biofilm and delivered via i/mam route was found to induce protective cell mediated immune

response against bovine mastitis in caused by *S. aureus* mouse model and reduced the colonization of *S. aureus* in mammary gland, severity of clinical symptoms and tissue damage in mammary gland of mice was lessened.

10.3 Future directions

(1) Given the finding of this project that biofilm forming *S. aureus* bacterins induce better antibody and cell-mediated immune responses and protection against intramammary challenge with the virulent pathogen, it is important to identify the nature of the biofilm- associated antigens and determine their relative contribution to the colonisation of mammary gland with a view to formulate the most effective vaccine capable of giving protection against bovine mastitis.

(2) Given the knowledge that production of high levels of interleukin 6 (IL-6) in mice challenged with non-typeable *S. aureus* isolates may have a role to play in per acute death of mice post-challenge as judged by a delay in the time of death upon administration of anti-IL-6 or anti IL-6Rβ antibodies, urgent attention on the prevention of mortality by treatment with a suitable antibiotic should also be an important priority.

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Appendix A

A.1 Buffers, Solutions and Media

1xPBS

Ingredients	Grams
NaCl	8
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24g

The mixture was dissolved in 800 ml of distilled water and PH was adjusted to 7.4. The volume was adjusted to 1L adding additional distilled water and autoclaved at 121°C for 15 minutes.

50 mM sodium acetate buffer

Ingredients	Millilitre
Acetic acid (0.2M)	7.4 ml
Sodium acetate (0.2 M)	17.6 ml

The volume was adjusted to 100 ml using distilled water.

50x TAE (Tris-acetate-EDTA) buffer

Ingredients	Gram/Millilitre
Tris base	22241 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M)	100 ml

The volume was adjusted to 1L by adding distilled water. To make 1xTAE 20ml of 50xTAE stock to 980 ml of distilled water.

0.5 M EDTA

93.5 g of Disodium ethylene diamine tetra-acetate, 2H20 was added to 400 ml of distilled water. The volume was adjusted to 500 ml by adding distilled water and autoclaved at 121°C for 15 minutes.

Agar rose gel (1.5%)

1.5g Agarose

100 ml 1X TAE buffer

Blocking buffer (ELISA)

Ingredients	**Gram/Millilitre**
Tris base	1.21 g
Tween-20	0.50 ml
1% PBS	999.50 ml

The stock was stored at 4ºC.

Carbonate bicarbonate coating buffer (ELISA)

Ingredients	**Gram/Millilitre**
$Na2CO_3$	1.59 g
$NaHCO_3$	2.94 g
Distilled water	900 ml

PH was adjusted to 9.6 and volume was adjusted to 1 L.

Congo red agar

Ingredients	**Gram/Millilitre**
Congo red	0.4 g
Glucose	10 g
Blood agar base	40 g
Distilled water	1L

Autoclaving was done at 121°C for 15 minutes.

Mueller Hinton agar

Ingredients	**Grams/Litre**
Beef infusion solids	4.0
Starch	1.5

Casein hydrolysate	17.5
Agar	15.0

Final pH was adjusted to 7.4.

Thirty eight gram of Mueller Hinton agar was suspended in 1 litre of distilled water, brought to the boil for complete dissolving of media and autoclaved at 121°C for 15 minutes.

NaCl (0.9%)

9 g of NaCl was dissolved in 1 L of distilled water and autoclaved at 121°C for 15 minutes.

Nutrient broth

Ingredients	**Grams/Litre**
Peptone	5.0
NaCl	5.0
Beef extract	1.5
Yeast extract	1.5

Thirteen gram of the mixture was suspended in 1L of distilled water and mixed thoroughly to dissolve the medium. Final pH was adjusted to 7.4 and autoclaved at 121°C for 15 minutes.

Sodium borate buffer (20X)

NaOH	8g
Boric acid	47g
Distilled water	900 ml

It was mixed thoroughly. The final volume was adjusted to 1 L by adding distilled water.

Substrate buffer (ELISA)

Ingredients	**Gram/Millilitre**
Diethanolamine	106 g
$MgSO_4$. $7H_2O$	0.249 g
Distilled water	800 ml

The PH was adjusted to 10 and the volume was made up to 1L using distilled water.

APPENDIX B

Complementary data for Chapters 3 to 9

B.1 Chapter 3

Table 1: List of samples used in the project

Sl.No	Sample	Species	Organism	Sample no
1	Milk	Bovine	*S.aureus*	BoAISRF-S.aur25
2	Milk	Bovine	*S.aureus*	BoAISRF-S.aur26
3	Milk	Bovine	*S.aureus*	BoAISRF-S.aur27
4	Milk	Bovine	*S.aureus*	BoAISRF-S.aur30
5	Milk	Bovine	*S.aureus*	BoAISRF-S.aur31
6	Milk	Bovine	*S.aureus*	BoAISRF-S.aur32
7	Milk	Bovine	*S.aureus*	BoAISRF-S.aur33
8	Milk	Bovine	*S.aureus*	BoAISRF-S.aur34
9	Milk	Bovine	*S.aureus*	BoAISRF-S.aur35
10	Milk	Bovine	*S.aureus*	BoAISRF-S.aur36
11	Milk	Bovine	*S.aureus*	BoAISRF-S.aur37
12	Milk	Bovine	*S.aureus*	BoAISRF-S.aur38
13	Milk	Bovine	*S.aureus*	BoAISRF-S.aur39
14	Milk	Bovine	*S.aureus*	BoAISRF-S.aur40
15	Milk	Bovine	*S.aureus*	BoAISRF-S.aur41
16	Milk	Bovine	*S.aureus*	BoAISRF-S.aur42
17	Milk	Bovine	*S.aureus*	BoAISRF-S.aur43
18	Milk	Bovine	*S.aureus*	BoAISRF-S.aur44
19	Milk	Bovine	*S.aureus*	BoAISRF-S.aur45
20	Milk	Bovine	*S.aureus*	BoAISRF-S.aur46
21	Milk	Bovine	*S.aureus*	BoAISRF-S.aur47
22	Milk	Bovine	*S.aureus*	BoAISRF-S.aur48
23	Milk	Bovine	*S.aureus*	BoAISRF-S.aur49
24	Milk	Bovine	*S.aureus*	BoAISRF-S.aur50
25	Milk	Bovine	*S.aureus*	BoAISRF-S.aur51
26	Milk	Bovine	*S.aureus*	BoAISRF-S.aur52
27	Milk	Bovine	*S.aureus*	BoAISRF-S.aur53
28	Milk	Bovine	*S.aureus*	BoAISRF-S.aur54
29	Milk	Bovine	*S.aureus*	BoAISRF-S.aur55
30	Milk	Bovine	*S.aureus*	BoAISRF-S.aur56
31	Milk	Bovine	*S.aureus*	BoAISRF-S.aur57
32	Milk	Bovine	*S.aureus*	BoAISRF-S.aur58
33	Milk	Bovine	*S.aureus*	BoAISRF-S.aur59
34	Milk	Bovine	*S.aureus*	BoAISRF-S.aur60
35	Milk	Bovine	*S.aureus*	BoAISRF-S.aur61
36	Milk	Bovine	*S.aureus*	BoAISRF-S.aur62
37	Milk	Bovine	*S.aureus*	BoAISRF-S.aur63
38	Milk	Bovine	*S.aureus*	BoAISRF-S.aur64
39	Milk	Bovine	*S.aureus*	BoAISRF-S.aur65
40	Milk	Bovine	*S.aureus*	BoAISRF-S.aur66

Sl.No	Sample	Species	Organism	Sample no
41	Milk	Bovine	*S.aureus*	BoAISRF-S.aur67
42	Milk	Bovine	*S.aureus*	BoAISRF-S.aur69
43	Milk	Bovine	*S.aureus*	BoAISRF-S.aur70
44	Milk	Bovine	*S.aureus*	BoAISRF -S.aur71
45	Milk	Bovine	*S.aureus*	BoAISRF -S.aur72
46	Milk	Bovine	*S.aureus*	BoAISRF -S.aur73
47	Milk	Bovine	*S.aureus*	BoAISRF -S.aur75
48	Milk	Bovine	*S.aureus*	BoAISRF -S.aur76
49	Milk	Bovine	*S.aureus*	BoAISRF -S.aur77
50	Milk	Bovine	*S.aureus*	BoAISRF -S.aur78
51	Milk	Bovine	*S.aureus*	BoAISRF -S.aur79
52	Milk	Bovine	*S.aureus*	BoAISRF -S.aur80
53	Milk	Bovine	*S.aureus*	BoAISRF-S.aur81
54	Milk	Bovine	*S.aureus*	BoAISRF-S.aur82
55	Milk	Bovine	*S.aureus*	BoAISRF-S.aur83
56	Milk	Bovine	*S.aureus*	BoAISRF-S.aur84
57	Milk	Bovine	*S.aureus*	BoAISRF-S.aur85
58	Milk	Bovine	*S.aureus*	BoAISRF-S.aur86
59	Milk	Bovine	*S.aureus*	BoAISRF-S.aur87
60	Milk	Bovine	*S.aureus*	BoAISRF -S.aur88
61	Milk	Bovine	*S.aureus*	BoAISRF-S.aur89
62	Milk	Bovine	*S.aureus*	BoAISRF-S.aur90
63	Milk	Bovine	*S.aureus*	BoAISRF-S.aur91
64	Milk	Bovine	*S.aureus*	BoAISRF-S.aur92
65	Milk	Bovine	*S.aureus*	BoAISRF-S.aur93
66	Milk	Bovine	*S.aureus*	BoAISRF-S.aur94
67	Milk	Bovine	*S.aureus*	BoAISRF-S.aur95
68	Milk	Bovine	*S.aureus*	BoAISRF-S.aur96
69	Milk	Bovine	*S.aureus*	BoAISRF-S.aur97
70	Milk	Bovine	*S.aureus*	BoAISRF-S.aur98
71	Milk	Bovine	*S.aureus*	BoAISRF-S.aur99
72	Milk	Bovine	*S.aureus*	BoAISRF-S.aur100
73	Milk	Bovine	*S.aureus*	BoAISRF-S.aur101
74	Milk	Bovine	*S.aureus*	BoAISRF-S.aur102
75	Milk	Bovine	*S.aureus*	BoAISRF-S.aur103
76	Milk	Bovine	*S.aureus*	BoAISRF-S.aur104
77	Milk	Bovine	*S.aureus*	BoAISRF-S.aur105
78	Milk	Bovine	*S.aureus*	BoAISRF-S.aur106
79	Milk	Bovine	*S.aureus*	BoAISRF-S.aur107
80	Milk	Bovine	*S.aureus*	BoAISRF-S.aur108
81	Milk	Bovine	*S.aureus*	BoAISRF-S.aur109
82	Milk	Bovine	*S.aureus*	BoAISRF-S.aur110
83	Milk	Bovine	*S.aureus*	BoAISRF-S.aur111
84	Milk	Bovine	*S.aureus*	BoAISRF-S.aur112
85	Milk	Bovine	*S.aureus*	BoAISRF-S.aur113
86	Milk	Bovine	*S.aureus*	BoAISRF-S.aur114
87	Milk	Bovine	*S.aureus*	BoAISRF-S.aur115

Sl.No	Sample	Species	Organism	Sample no
88	Milk	Bovine	*S.aureus*	BoAISRF-S.aur116
89	Milk	Bovine	*S.aureus*	BoAISRF-S.aur117
90	Milk	Bovine	*S.aureus*	BoAISRF-S.aur118
91	Milk	Bovine	*S.aureus*	BoAISRF-S.aur119
92	Milk	Bovine	*S.aureus*	BoAISRF-S.aur120
93	Milk	Bovine	*S.aureus*	BoAISRF-S.aur121
94	Milk	Bovine	*S.aureus*	BoAISRF-S.aur122
95	Milk	Bovine	*S.aureus*	BoAISRF-S.aur123
96	Milk	Bovine	*S.aureus*	BoAISRF-S.aur124
97	Milk	Bovine	*S.aureus*	BoAISRF-S.aur125
98	Milk	Bovine	*S.aureus*	BoAISRF-S.aur126
99	Milk	Bovine	*S.aureus*	BoAISRF-S.aur127
100	Milk	Bovine	*S.aureus*	BoAISRF-S.aur128
101	Milk	Bovine	*S.aureus*	BoAISRF-S.aur129
102	Milk	Bovine	*S.aureus*	BoAISRF-S.aur130
103	Milk	Bovine	*S.aureus*	BoAISRF-S.aur131
104	Milk	Bovine	*S.aureus*	BoAISRF-S.aur132
105	Milk	Bovine	*S.aureus*	BoAISRF-S.aur133
106	Milk	Bovine	*S.aureus*	BoAISRF-S.aur134
107	Milk	Bovine	*S.aureus*	BoAISRF-S.aur135
108	Milk	Bovine	*S.aureus*	BoAISRF-S.aur136
109	Milk	Bovine	*S.aureus*	BoAISRF-S.aur137
110	Milk	Bovine	*S.aureus*	BoAISRF-S.aur138
111	Milk	Bovine	*S.aureus*	BoAISRF-S.aur139
112	Milk	Bovine	*S.aureus*	BoAISRF-S.aur140
113	Milk	Bovine	*S.aureus*	BoAISRF-S.aur141
114	Milk	Bovine	*S.aureus*	BoAISRF-S.aur142
115	Milk	Bovine	*S.aureus*	BoAISRF-S.aur143
116	Milk	Bovine	*S.aureus*	BoAISRF-S.aur144
117	Milk	Bovine	*S.aureus*	BoAISRF-S.aur145
118	Milk	Bovine	*S.aureus*	BoAISRF-S.aur146
119	Milk	Bovine	*S.aureus*	BoAISRF-S.aur147
120	Milk	Bovine	*S.aureus*	BoAISRF-S.aur148
121	Milk	Bovine	*S.aureus*	BoAISRF-S.aur149
122	Milk	Bovine	*S.aureus*	BoAISRF-S.aur150
123	Milk	Bovine	*S.aureus*	BoAISRF-S.aur151
124	Milk	Bovine	*S.aureus*	BoAISRF-S.aur152
125	Milk	Bovine	*S.aureus*	BoAISRF-S.aur153
126	Milk	Bovine	*S.aureus*	BoAISRF-S.aur154
127	Milk	Bovine	*S.aureus*	BoAISRF-S.aur155
128	Milk	Bovine	*S.aureus*	BoAISRF-S.aur156
129	Milk	Bovine	*S.aureus*	BoAISRF-S.aur157
130	Milk	Bovine	*S.aureus*	BoAISRF-S.aur158
131	Milk	Bovine	*S.aureus*	BoAISRF-S.aur159
132	Milk	Bovine	*S.aureus*	BoAISRF-S.aur160
133	Milk	Bovine	*S.aureus*	BoAISRF-S.aur161
134	Milk	Bovine	*S.aureus*	BoAISRF-S.aur162

Sl.No	Sample	Species	Organism	Sample no
135	Milk	Bovine	*S.aureus*	BoAISRF-S.aur163
136	Milk	Bovine	*S.aureus*	BoAISRF-S.aur165
137	Milk	Bovine	*S.aureus*	BoAISRF-S.aur166
138	Milk	Bovine	*S.aureus*	BoAISRF-S.aur167
139	Milk	Bovine	*S.aureus*	BoAISRF-S.aur168
140	Milk	Bovine	*S.aureus*	BoAISRF-S.aur169
141	Milk	Bovine	*S.aureus*	BoAISRF-S.aur170
142	Milk	Bovine	*S.aureus*	BoAISRF-S.aur171
143	Milk	Bovine	*S.aureus*	BoAISRF-S.aur172
144	Milk	Bovine	*S.aureus*	BoAISRF-S.aur173
145	Milk	Bovine	*S.aureus*	BoAISRF-S.aur174
146	Milk	Bovine	*S.aureus*	BoAISRF-S.aur175
147	Milk	Bovine	*S.aureus*	BoAISRF-S.aur176
148	Milk	Bovine	*S.aureus*	BoAISRF-S.aur177
149	Milk	Bovine	*S.aureus*	BoAISRF-S.aur178
150	Milk	Bovine	*S.aureus*	BoAISRF-S.aur179
151	Milk	Bovine	*S.aureus*	BoAISRF-S.aur180
152	Milk	Bovine	*S.aureus*	BoAISRF-S.aur181
153	Milk	Bovine	*S.aureus*	BoAISRF-S.aur182
154	Milk	Bovine	*S.aureus*	BoAISRF-S.aur183

Table 2: List of strains used as positive controls in the project

Sl.No	Species	Organism	Sample no
1	Human	*S.aureus*	Strain M (CP1)
2	Human	*S.aureus*	Strain Smith Diffuse (CP2)
3	Human	*S.aureus*	Strain Newman (CP5)
4	Human	*S.aureus*	NRS 648 (CP5)
5	Human	*S.aureus*	USA 400 (CP8)
6	Human	*S.aureus*	LAC (Non-Capsulated)
7	Human	*S.aureus*	NRS 680 (Non-Capsulated)

Table 3: Production of biofilm and detection of *icaA* and *icaD* genes in 154 bovine *S. aureus* isolates

BOAISRF S. aur Strain	*icaA*	*icaD*	OD value (TCP method)
25	✓	✓	0.865
26	✓	✓	0.548
27	✓	✓	0.346
30	✓	✓	0.406
31	✓	✓	0.474
32	✓	✓	0.515
33	✓	✓	0.206
34	✓	✓	0.558
35	✓	✓	0.941
36	✓	✓	0.455
37	✓	−	0.985
38	✓	✓	0.332
39	✓	✓	0.394
40	✓	✓	0.597
41	−	✓	1.413
42	✓	✓	0.222
43	✓	−	0.878
44	✓	−	0.347
45	✓	−	1.839
46	✓	✓	0.684
47	✓	✓	0.611
48	✓	✓	0.881
49	✓	✓	0.64
50	✓	✓	0.779
51	✓	✓	1.298
52	−	✓	0.339
53	−	✓	0.494
54	✓	✓	0.528
55	✓	✓	0.482
56	✓	✓	0.344
57	−	✓	0.22
58	✓	✓	0.284
59	✓	✓	0.448
60	✓	✓	1.162
61	✓	✓	0.574
62	✓	✓	0.491
63	✓	✓	0.443

BOAISRF S. aur Strain	***icaA***	***icaD***	**OD value (TCP method)**
64	✓	✓	0.26
65	✓	✓	0.741
66	✓	✓	0.434
67	✓	✓	0.342
69	✓	✓	0.556
70	–	✓	0.217
71	✓	✓	0.561
72	✓	✓	0.424
73	✓	✓	0.572
75	–	✓	1.022
76	–	✓	0.712
77	✓	✓	0.42
78	–	✓	0.799
79	–	–	0.302
80	–	–	0.907
81	–	–	0.289
82	–	✓	0.468
83	✓	✓	0.775
84	–	✓	0.251
85	–	–	0.507
86	–	✓	0.404
87	–	–	0.367
88	–	✓	0.243
89	–	–	0.328
90	–	–	0.468
91	–	✓	0.725
92	–	–	0.251
93	–	✓	0.507
94	–	✓	0.404
95	–	✓	0.368
96	✓	✓	0.243
97	✓	✓	0.44
98	✓	✓	0.313
99	✓	✓	0.632
100	✓	✓	0.301
101	✓	✓	0.448
102	✓	✓	0.172
103	✓	✓	0.825
104	–	–	0.494
105	✓	✓	0.818
106	✓	–	0.615
107	✓	✓	0.295

BOAISRF S. aur Strain	*icaA*	*icaD*	OD value (TCP method)
108	✓	✓	0.514
109	✓	✓	0.355
110	✓	✓	0.613
111	✓	✓	0.483
112	✓	✓	0.754
113	−	✓	0.24
114	✓	✓	0.679
115	✓	✓	0.352
116	✓	✓	0.239
117	✓	✓	0.194
118	✓	✓	0.259
119	✓	✓	0.172
120	✓	✓	0.359
121	✓	✓	0.34
122	✓	✓	0.63
123	✓	✓	0.777
124	✓	✓	0.228
125	✓	✓	0.231
126	✓	✓	0.246
127	✓	✓	0.248
128	✓	✓	0.304
129	−	−	0.345
130	−	−	0.427
131	−	✓	0.325
132	−	−	0.225
133	✓	✓	0.335
134	−	✓	0.294
135	✓	✓	0.143
136	✓	✓	0.402
137	✓	✓	0.398
138	✓	✓	0.24
139	✓	✓	0.446
140	✓	✓	0.425
141	−	✓	0.264
142	✓	✓	0.235
143	✓	✓	0.303
144	✓	✓	0.556
145	−	✓	0.405
146	−	✓	0.273
147	−	−	0.268
148	✓	✓	0.428

BOAISRF S. aur Strain	*icaA*	*icaD*	OD value (TCP method)
149	✓	✓	0.208
150	−	✓	0.13
151	✓	✓	0.292
152	✓	✓	0.301
153	✓	✓	0.393
154	✓	✓	0.295
155	−	✓	0.237
156	✓	✓	0.191
157	✓	✓	0.183
158	✓	✓	0.258
159	✓	✓	0.316
160	✓	✓	0.511
161	✓	✓	0.298
162	✓	✓	0.432
163	✓	✓	0.287
165	−	✓	0.093
166	✓	✓	0.199
167	✓	✓	0.488
168	−	✓	0.297
169	−	−	0.162
170	−	−	0.256
171	−	−	0.221
172	−	−	0.137
173	−	−	0.364
174	✓	✓	0.426
175	−	−	0.275
176	✓	✓	0.295
177	−	−	0.408
178	✓	✓	0.269
179	✓	✓	0.233
180	✓	−	0.254
181	✓	✓	0.491
182	✓	✓	0.283
183	✓	✓	0.276

The arbitrary cut-off point used for nonbiofilm-producing strains was 0.120 (Stepanovic et al., 2000). The arbitrary cut-off points used for weakly, intermediate or strongly adherent bacterial populations were 0.130–4.00×0.120 A570nm, 4.10–5.90×0.120 A570nm and greater than 6×0.120 A570nm, respectively.

Table 4: Detection of *bap, blaz* and *agr* types in 154 bovine *S. aureus* isolates

***S.aureus* strain**	***bap* gene**	***blaz* gene**	***agr* type**
25	-	+	I
26	-	-	I
27	-	-	-
30	-	-	I
31	-	-	III
32	-	-	III
33	-	-	III
34	-	-	III
35	-	-	I
36	-	+	I
37	-	+	I
38	-	+	I
39	-	+	I
40	-	-	
41	-	-	III
42	-	-	-
43	-	-	I
44	-	-	I
45	-	-	I
46	-	-	III
47	-	-	III
48	-	-	I
49	-	-	III
50	-	-	III
51	-	-	III
52	-	-	III
53	-	-	-

***S.aureus* strain**	***bap* gene**	***blaz* gene**	***agr* type**
54	-	-	-
55	-	-	-
56	-	-	I
57	-	-	I
58	-	-	-
59	-	+	I
60	-	-	I
61	-	-	III
62	-	-	I
63	-	+	-
64	-	-	I
65	-	-	III
66	-	-	I
67	-	-	I
69	-	-	I
70	-	-	-
71	-	-	-
72	-	-	I
73	-	-	III
75	-	-	III
76	-	-	III
77	-	+	I
78	-	+	I
79	-	-	II
80	-	-	III
81	-	-	I
82	-	+	III
83	-	-	-
84	-	-	I
85	-	-	I

***S.aureus* strain**	***bap* gene**	***blaz* gene**	***agr* type**
86	-	-	I
87	-	-	I
88	-	-	I
89	-	-	I
90	-	-	I
91	-	-	I
92	-	-	III
93	-	-	I
94	-	-	I
95	-	-	I
96	-	-	I
97	-	-	-
98	-	-	I
99	-	+	I
100	-	-	I
101	-	-	I
102	-	-	III
103	-	-	I
104	-	-	-
105	-	-	I
106	-	-	I
107	-	-	I
108	-	-	I
109	-	-	I
110	-	-	I
111	-	-	III
112	-	-	I
113	-	+	I
114	-	-	II
115	-	-	III

S.aureus **strain**	***bap*** **gene**	***blaz*** **gene**	***agr*** **type**
116	-	-	III
117	-	+	I
118	-	+	III
119	-	-	III
120	-	-	I
121	-	-	I
122	-	-	I
123	-	-	I
124	-	-	-
125	-	-	I
126	-	-	III
127	-	-	-
128	-	-	III
129	-	-	I
130	-	-	I
131	-	-	I & III
132	-	-	I
133	-	+	I
134	-	-	I
135	-	+	III
136	-	+	III
137	-	-	I
138	-	+	I
139	-	-	I
140	-	+	I
141	-	+	III
142	-	-	I
143	-	-	I
144	-	-	I
145	-	-	I

S.aureus **strain**	***bap*** **gene**	***blaz*** **gene**	***agr*** **type**
146	+	-	-
147	-	+	-
148	-	-	III
149	-	-	III
150	-	-	I
151	-	+	-
152	-	-	III
153	-	-	I
154	-	-	I
155	-	-	I
156	-	-	III
157	-	-	III
158	-	-	III
159	-	-	I
160	-	-	I
161	-	-	III
162	-	-	I
163	-	-	III
165	-	+	-
166	-	+	III
167	-	+	III
168	-	+	-
169	-	-	-
170	-	-	-
171	-	+	-
172	-	-	-
173	-	+	I
174	-	+	III
175	-	-	-
176	-	+	III

S.aureus **strain**	***bap*** **gene**	***blaz*** **gene**	***agr*** **type**
177	-	-	-
178	-	+	III
179	-	-	III
180	-	-	III
181	-	-	-
182	-	-	-
183	+	-	I

B 2: Chapter 4

Table 1: Detection of various toxin genes in 154 bovine S. aureus isolates

S. aureus strain	α-Toxin	β-Toxin	SEA	SEB	SEC	SED	SEE	SEG	SHE	SEI	SEJ
25	+	+	-	-	+	-	-	+	-	+	-
26	+	+	-	-	-	-	-	-	-	-	-
27	+	-	-	-	-	-	-	-	-	-	-
30	+	+	-	-	-	-	-	-	-	-	-
31	+	+	-	-	-	-	-	-	+	-	-
32	+	+	-	-	-	-	-	-	+	-	-
33	+	+	-	-	-	-	-	-	+	-	-
34	+	+	-	-	-	-	-	-	+	-	-
35	+	+	-	-	-	-	-	-	-	-	-
36	+	+	-	-	-	-	-	-	-	-	-
37	+	+	-	-	-	-	-	-	-	-	-
38	+	+	-	-	-	-	-	-	-	-	-
39	+	+	-	-	-	-	-	-	-	-	-
40	+	+	-	-	+	-	-	+	-	+	-
41	+	+	-	-	-	-	-	-	+	-	-
42	+	+	-	-	+	-	-	+	-	+	-
43	+	+	-	-	+	-	-	-	-	-	-
44	+	+	-	-	+	-	-	-	-	-	-
45	+	+	-	-	+	-	-	-	-	-	-
46	+	+	-	-	+	-	-	-	+	-	-

S. aureus strain	α-Toxin	β-Toxin	SEA	SEB	SEC	SED	SEE	SEG	SHE	SEI	SEJ
47	+	+	-	-	-	-	-	-	+	-	-
48	+	+	-	-	-	-	-	-	-	-	-
49	+	+	-	-	-	-	-	-	+	-	-
50	+	+	-	-	-	-	-	-	+	-	-
51	+	+	-	-	-	-	-	-	+	-	-
52	+	-	-	-	-	-	-	-	+	-	-
53	+	+	-	-	-	-	-	-	+	-	-
54	+	-	-	-	+	-	-	+	-	+	-
55	+	+	-	-	-	-	-	-	+	-	-
56	+	+	-	-	-	-	-	-	-	-	-
57	+	+	-	-	-	-	-	-	-	-	-
58	+	-	-	-	-	-	-	+	-	+	-
59	+	+	-	-	-	-	-	-	-	-	-
60	+	-	-	-	-	-	-	-	-	-	-
61	+	+	-	-	-	-	-	-	+	-	-
62	+	+	-	-	-	-	-	-	-	-	-
63	+	-	-	-	-	-	-	-	-	-	-
64	+	+	-	-	-	-	-	-	-	-	-
65	+	+	-	-	-	-	-	-	+	-	-
66	+	+	-	-	-	-	-	-	-	-	-
67	-	+	-	-	-	-	-	-	-	-	-
69	+	+	-	-	-	-	-	-	-	-	-
70	+	-	-	-	-	-	-	+	-	+	-
71	+	+	-	-	+	-	-	+	-	+	-
72	+	+	-	-	-	-	-	-	-	-	-

S. aureus strain	α-Toxin	β-Toxin	SEA	SEB	SEC	SED	SEE	SEG	SHE	SEI	SEJ
73	+	-	-	-	-	-	-	-	+	-	-
75	-	+	-	-	-	-	-	-	+	-	-
76	+	+	-	-	-	-	-	-	+	-	-
77	+	+	-	-	-	-	-	-	-	-	-
78	+	+	-	-	-	-	-	-	-	-	-
79	+	+	-	-	-	+	-	+	-	+	-
80	+	+	-	-	+	-	-	-	+	-	-
81	+	+	-	-	-	-	-	-	-	-	-
82	+	+	-	-	-	-	-	-	+	-	-
83	+	+	-	-	+	+	-	+	-	+	-
84	+	+	-	-	-	-	-	-	-	+	-
85	+	+	-	-	-	-	-	-	-	-	-
86	+	+	-	-	-	-	-	-	-	-	-
87	+	+	-	-	-	-	-	-	-	-	-
88	+	+	-	-	-	-	-	-	-	-	-
89	+	-	-	-	-	-	-	-	-	-	-
90	+	+	-	-	-	-	-	-	-	-	-
91	+	-	-	-	-	-	-	-	+	-	-
92	+	+	-	-	-	-	-	-	+	-	-
93	+	+	-	-	-	-	-	-	-	-	-
94	+	+	-	-	-	-	-	-	+	-	-
95	+	+	-	-	-	-	-	-	-	-	-
96	+	+	-	-	-	-	-	-	-	-	+
97	+	-	-	-	-	-	-	-	-	-	-
98	+	+	-	-	-	-	-	-	+	-	-

S. aureus strain	α-Toxin	β-Toxin	SEA	SEB	SEC	SED	SEE	SEG	SHE	SEI	SEJ
99	+	+	-	-	-	-	-	-	+	-	-
100	+	-	-	-	-	-	-	-	-	-	-
101	+	+	-	-	-	-	-	-	-	-	-
102	+	+	-	-	-	-	-	-	+	-	-
103	+	+	-	-	-	-	-	-	-	-	-
104	+	+	-	-	-	-	-	-	-	-	-
105	+	+	-	-	-	-	-	-	-	-	-
106	+	+	-	-	-	-	-	-	-	-	-
107	+	+	-	-	-	-	-	-	-	-	-
108	+	+	-	-	-	-	-	-	-	-	-
109	+	+	-	-	-	-	-	-	-	-	-
110	+	+	-	-	-	-	-	+	-	-	-
111	+	+	-	-	+	-	-	-	+	-	-
112	+	+	-	-	-	-	-	-	-	-	-
113	+	+	-	-	+	-	-	-	-	-	-
114	+	+	-	-	+	-	-	+	-	+	-
115	+	+	-	-	+	-	-	+	+	-	-
116	+	-	-	-	+	-	-	+	+	-	-
117	+	+	-	-	-	-	-	+	+	+	-
118	+	+	-	-	-	-	-	+	+	+	-
119	+	+	-	-	-	-	-	-	+	-	-
120	+	+	-	-	-	-	-	-	-	-	-
121	+	+	-	-	-	-	-	-	-	-	-
122	+	+	-	-	-	-	-	-	-	-	-
123	+	+	-	-	+	-	-	-	-	-	-

S. aureus strain	α-Toxin	β-Toxin	SEA	SEB	SEC	SED	SEE	SEG	SHE	SEI	SEJ
124	+	+	-	-	+	-	-	+	-	+	-
125	+	+	+	-	-	-	-	+	-	-	+
126	+	-	-	-	-	-	-	-	+	-	-
127	+	+	-	-	+	-	-	+	-	+	-
128	+	-	-	-	+	-	-	-	+	-	-
129	+	+	-	-	-	-	-	-	-	-	-
130	+	+	-	-	-	-	-	-	-	-	-
131	+	+	-	-	-	-	-	-	-	-	-
132	+	+	-	-	-	-	-	-	-	-	-
133	+	+	-	-	+	-	-	-	+	-	-
134	+	+	-	-	+	-	-	-	-	-	-
135	+	-	-	-	+	-	-	+	-	-	-
136	+	+	-	-	+	-	-	+	+	-	-
137	+	+	-	-	+	-	-	-	-	-	-
138	+	+	-	-	+	-	-	-	-	-	-
139	+	+	-	-	-	-	-	+	-	-	-
140	+	+	-	-	-	-	-	-	-	-	-
141	+	+	-	-	+	-	-	+	-	-	-
142	+	+	-	-	-	-	-	-	-	+	-
143	+	+	-	-	+	-	-	+	-	-	-
144	+	+	-	-	+	-	-	-	-	+	-
145	+	+	-	-	+	-	-	-	-	-	-
146	+	+	-	-	-	-	-	+	-	-	-
147	+	+	+	-	-	-	+	-	+	-	-
148	+	+	-	-	-	-	-	-	+	-	-

S. aureus strain	α-Toxin	β-Toxin	SEA	SEB	SEC	SED	SEE	SEG	SHE	SEI	SEJ
149	+	+	-	-	-	-	-	-	+	-	-
150	+	+	+	-	-	-	-	-	-	-	-
151	-	+	+	+	-	-	-	-	-	-	-
152	+	+	-	-	-	-	-	-	+	-	-
153	+	+	-	-	-	-	-	-	-	-	-
154	+	+	-	-	-	-	-	-	-	-	-
155	+	-	-	-	-	-	-	-	-	-	-
156	+	+	-	-	-	-	-	-	+	-	-
157	+	+	-	-	-	-	-	-	-	-	-
158	+	+	-	-	-	-	-	-	+	-	-
159	+	+	-	-	-	-	-	-	-	-	-
160	+	+	-	-	-	-	-	-	-	-	-
161	+	+	-	-	-	-	-	-	-	-	-
162	+	+	-	-	-	-	-	-	+	-	-
163	+	+	-	-	-	-	-	-	-	-	-
165	-	-	-	-	-	-	-	-	-	-	-
166	+	+	-	-	-	-	-	-	-	-	-
167	+	-	-	-	-	-	-	-	-	-	-
168	+	+	-	-	-	-	-	-	-	-	-
169	+	-	-	-	-	-	-	-	-	-	-
170	-	+	-	-	-	-	-	-	-	-	-
171	-	-	-	-	-	-	-	-	-	+	-
172	+	-	-	-	-	-	-	-	-	-	-
173	-	-	-	-	-	-	-	-	-	-	-
174	+	+	-	-	-	-	-	-	-	-	-

S. aureus strain	α-Toxin	β-Toxin	SEA	SEB	SEC	SED	SEE	SEG	SHE	SEI	SEJ
175	-	-	-	-	-	-	-	-	-	-	-
176	+	+	-	-	-	-	-	-	-	-	-
177	+	-	-	-	-	-	-	-	-	-	-
178	-	+	-	-	+	-	-	-	-	-	-
179	+	+	-	-	+	-	-	-	+	+	-
180	+	+	-	-	+	-	-	-	+	-	-
181	+	-	-	-	+	-	-	+	+	-	-
182	+	+	-	-	+	-	-	+	+	+	-
183	+	+	-	-	+	-	-	+	+	-	-

Table 2: Detection of MSCRAMMS genes in 154 bovine *S. aureus* isolates

S. aureus strain	FnBPb	FnBPa	SpA	Cna	ClfA	ClfB	bbp	isdA	isdB	sdrD	sdrE
25	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
26	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
27	✓	-	✓	-	✓	✓	-	✓	✓	-	✓
30	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
31	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
32	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
33	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
34	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
35	-	✓	✓	-	-	✓	-	✓	✓	✓	✓
36	-	✓	✓	-	-	✓	-	✓	✓	✓	✓
37	-	✓	✓	-	-	✓	-	✓	✓	✓	✓
38	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
39	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
40	-	✓	✓	-	✓	✓	✓	✓	✓	✓	✓
41	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
42	-	-	✓	-	✓	✓	✓	✓	✓	✓	✓
43	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
44	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
45	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
46	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓

S. aureus strain	FnBPb	FnBPa	SpA	Cna	ClfA	ClfB	bbp	isdA	isdB	sdrD	sdrE
47	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
48	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
49	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
50	-	✓	✓	✓	✓	✓	-	✓	✓	✓	-
51	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
52	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
53	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
54	-	✓	✓	-	✓	✓	✓	✓	✓	✓	✓
55	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
56	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
57	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
58	-	-	✓	-	✓	✓	✓	✓	✓	-	✓
59	-	✓	✓	-	✓	✓	-	✓	✓	✓	-
60	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
61	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
62	-	-	✓	✓	✓	✓	-	-	✓	✓	✓
63	-	✓	✓	-	✓	✓	-	-	✓	✓	✓
64	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
65	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
66	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
67	-	✓	✓	-	✓	-	-	✓	✓	✓	✓
69	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
70	-	✓	✓	-	✓	✓	✓	✓	✓	✓	✓
71	-	✓	✓	-	✓	✓	✓	✓	✓	✓	✓

S. aureus strain	FnBPb	FnBPa	SpA	Cna	ClfA	ClfB	bbp	isdA	isdB	sdrD	sdrE
72	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
73	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
75	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
76	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
77	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
78	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
79	-	-		-	✓	✓	✓	✓	✓	✓	✓
80	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
81	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
82	-	-	-	-	✓	✓	-	✓	✓	✓	✓
83	-	-	✓	-	✓	✓	✓	✓	✓	✓	✓
84	-	-	-	-	✓	✓	-	✓	✓	✓	✓
85	-	-	-	-	✓	✓	-	✓	✓	✓	✓
86	-	-	-	-	-	✓	-	✓	✓	✓	✓
87	-	-	-	-	✓	✓	-	✓	✓	✓	✓
88	-	-	-	-	✓	✓	-	✓	✓	✓	✓
89	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
90	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
91	-	-	-	-	✓	✓	-	✓	✓	✓	✓
92	-	-	-	✓	✓	✓	-	✓	✓	✓	✓
93	-	-	-	-	✓	✓	-	✓	✓	✓	-
94	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
95	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
96	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓

S. aureus strain	FnBPb	FnBPa	SpA	Cna	ClfA	ClfB	bbp	isdA	isdB	sdrD	sdrE
97	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
98	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
99	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
100	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
101	-	-	✓	-	✓	✓	-	✓	-	✓	✓
102	-	✓	-	✓	✓	✓	-	✓	✓	✓	✓
103	-	✓	-	-	✓	✓	-	✓	✓	✓	✓
104	-	-	-	✓	-	✓	-	✓	✓	✓	✓
105	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
106	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
107	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
108	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
109	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
110	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
111	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
112	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
113	-	✓	✓	✓	✓	✓	-	✓	✓	✓	-
114	-	✓	✓	-	✓	✓	✓	✓	✓	✓	✓
115	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
116	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
117	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
118	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
119	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
120	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓

S. aureus strain	FnBPb	FnBPa	SpA	Cna	ClfA	ClfB	bbp	isdA	isdB	sdrD	sdrE
121	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
122	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
123	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
124	-	-	✓	-	✓	✓	✓	✓	✓	✓	✓
125	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
126	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
127	-	-	✓	-	✓	✓	✓	✓	✓	✓	✓
128	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
129	-	-	-	-	✓	✓	-	✓	✓	✓	✓
130	-	-	-	-	✓	✓	-	✓	✓	✓	✓
131	-	-	-	-	✓	✓	-	✓	✓	✓	✓
132	-	-	-	-	✓	✓	-	✓	✓	✓	✓
133	-	-	✓	✓	✓	✓	-	-	✓	✓	✓
134	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
135	-	-	-	-	✓	✓	-	✓	✓	✓	✓
136	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
137	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
138	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
139	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
140	✓	-	✓	-	✓	✓	-	✓	✓	✓	✓
141	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
142	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
143	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
144	-	-	✓	-	✓	✓	-	✓	✓	✓	✓

S. aureus strain	FnBPb	FnBPa	SpA	Cna	ClfA	ClfB	bbp	isdA	isdB	sdrD	sdrE
145	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
146	-	-	✓	-	-	-	-	✓	✓	✓	-
147	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
148	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
149	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
150	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
151	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
152	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
153	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
154	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
155	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
156	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
157	-	-	✓	✓	✓	✓	-	✓	✓	✓	✓
158	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
159	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
160	-	-	✓	✓	✓	✓	-	✓	-	✓	✓
161	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
162	-	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
163	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
165	-	-	✓	-	✓	-	-	✓	✓	✓	-
166	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
167	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
168	-	-	✓	-	✓	-	-	✓	✓	✓	-
169	-	-	✓	-	-	-	-	✓	✓	✓	✓

S. aureus strain	FnBPb	FnBPa	SpA	Cna	ClfA	ClfB	bbp	isdA	isdB	sdrD	sdrE
170	-	-	✓	-	-	-	-	✓	✓	✓	✓
171	-	-	✓	-	-	-	-	✓	✓	✓	✓
172	-	-	✓	-	-	-	-	✓	✓	✓	✓
173	-	-	✓	-	-	-	✓	✓	✓	✓	✓
174	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
175	-	-	✓	-	-	-	-	✓	✓	✓	✓
176	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
177	-	-	✓	-	-	-	-	✓	✓	✓	✓
178	-	-	✓	-	✓	✓	-	✓	✓	✓	✓
179	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
180	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
181	-	-		-	✓	✓	✓	✓	✓	-	✓
182	-	✓	✓	-	✓	✓	-	✓	✓	✓	✓
183	-	✓	✓	-	✓	✓	✓	✓	✓	✓	✓

B. 3: Chapter 5

Table 1: Table 5.1: Prevalence of capsular/surface polysaccharide genotypes and phenotypes in 154 bovine *S. aureus* isolates

Sl. No	*S aureus* bovine strain	Detection of capsular genotype by PCR method					Detection of capsular serotype by Slide Agglutination test			
		CP1	CP2	CP5	CP8	CP1	CP2	CP5	CP8	SP336
1	BoAISRF-S.aur25	-	-	-	-	-	√	-	-	-
2	BoAISRF-S.aur26	-	-	-	√	-	-	-	√	-
3	BoAISRF-S.aur27	-	-	-	√	-	-	-	√	-
4	BoAISRF-S.aur30	-	-	√	-	-	-	√	-	-
5	BoAISRF-S.aur31	-	-	-	√	-	-	-	√	-
6	BoAISRF-S.aur32	-	-	-	√	-	-	-	√	-
7	BoAISRF-S.aur33	-	-	-	√	-	-	-	√	-
8	BoAISRF-S.aur34	-	-	-	√	-	-	-	√	-
9	BoAISRF-S.aur35	-	-	√	-	-	-	√	-	-
10	BoAISRF-S.aur36	-	-	√	-	-	-	√	-	-
11	BoAISRF-S.aur37	-	-	√	-	-	-	√	-	-
12	BoAISRF-S.aur38	-	-	√	-	-	-	√	-	-
13	BoAISRF-S.aur39	-	-	√	-	-	-	√	-	-
14	BoAISRF-S.aur40	-	-	√	-	-	-	√	-	-
15	BoAISRF-S.aur41	-	-	-	√	-	-	-	√	-
16	BoAISRF-S.aur42	-	-	-	√	-	-	-	√	-
17	BoAISRF-S.aur43	-	-	-	√	-	-	-	√	-
18	BoAISRF-S.aur44	-	-	√	-	-	-	√	-	-
19	BoAISRF-S.aur45	-	-	-	-	-	-	-	-	√

20	BoAISRF-S.aur46	-	-	-	√	-	-	-	√	-
21	BoAISRF-S.aur47	-	-	-	√	-	-	-	√	-
22	BoAISRF-S.aur48	-	-	-	√	-	-	-	√	-
23	BoAISRF-S.aur49	-	-	√	-	-	-	√	-	-
24	BoAISRF-S.aur50	-	-	-	√	-	-	-	√	-
25	BoAISRF-S.aur51	-	-	-	√	-	-	-	√	-
26	BoAISRF-S.aur52	-	-	-	-	-	√	-	-	-
27	BoAISRF-S.aur53	-	-	-	√	-	-	-	√	-
28	BoAISRF-S.aur54	-	-	-	√	-	-	-	√	-
29	BoAISRF-S.aur55	-	-	-	√	-	-	-	√	-
30	BoAISRF-S.aur56	-	-	-	√	-	-	-	√	-
31	BoAISRF-S.aur57	-	-	-	-	-	-	-	-	-
32	BoAISRF-S.aur58	-	-	-	√	-	-	-	√	-
33	BoAISRF-S.aur59	-	-	√	-	-	-	√	-	-
34	BoAISRF-S.aur60	-	-	√	-	-	-	-	-	-
35	BoAISRF-S.aur61	-	-	-	√	-	-	-	√	-
36	BoAISRF-S.aur62	-	-	-	-	-	-	-	-	-
37	BoAISRF-S.aur63	-	-	-	√	-	-	-	√	-
38	BoAISRF-S.aur64	-	-	√	-	-	-	-	-	-
39	BoAISRF-S.aur65	-	-	-	√	-	-	-	√	-
40	BoAISRF-S.aur66	-	-	-	-	-	-	-	-	-
41	BoAISRF-S.aur67	-	-	√	-	-	-	√	-	-
42	BoAISRF-S.aur69	-	-	-	-	-	-	-	-	-
43	BoAISRF-S.aur70	-	-	-	√	-	-	-	√	-
44	BoAISRF -.aur71	-	-	-	√	-	-	-	√	-
45	BoAISRF-S.aur72	-	-	-	√	-	-	-	√	-

46	BoAISRF-S.aur73	-	-	-	√	-	-	-	√	-
47	BoAISR -S.aur75	-	-	-	√	-	-	-	√	-
48	BoAISRF-S.aur76	-	-	-	√	-	-	-	√	-
49	BoAISRF-S.aur77	-	-	√	-	-	-	√	-	-
50	BoAISRF-S.aur78	-	-	√	-	-	-	√	-	-
51	BoAISRF-S.aur79	-	-	-	-	-	√	-	-	-
52	BoAISRF-S.aur80	-	-	-	-	-	√	-	-	-
53	BoAISRF-S.aur81	-	-	-	-	-	√	-	-	-
54	BoAISRF-S.aur82	-	-	-	-	-	-	-	-	√
55	BoAISRF-S.aur83	-	-	-	-	-	-	-	-	-
56	BoAISRF-S.aur84	-	-	-	-	-	-	-	-	-
57	BoAISRF-S.aur85	-	-	-	-	-	-	-	-	-
58	BoAISRF-S.aur86	-	-	-	-	-	-	-	-	√
59	BoAISRF-S.aur87	-	-	-	-	-	-	-	-	-
60	BoAISRF-S.aur88	-	-	-	-	-	√	-	-	-
61	BoAISRF-S.aur89	-	-	-	-	-	-	-	-	-
62	BoAISRF-S.aur90	-	-	-	-	-	-	-	-	-
63	BoAISRF-S.aur91	-	-	-	-	-	√	-	-	-
64	BoAISRF-S.aur92	-	-	-	-	-	-	-	-	-
65	BoAISRF-S.aur93	-	-	-	-	-	-	-	-	√
66	BoAISRF-S.aur94	-	-	-	-	-	-	-	-	-
67	BoAISRF-S.aur95	-	-	-	-	-	-	-	-	-
68	BoAISRF-S.aur96	-	-	-	-	-	-	-	-	-
69	BoAISRF-S.aur97	-	-	-	-	-	-	-	-	√
70	BoAISRF-S.aur98	-	-	-	-	-	-	-	-	-
71	BoAISRF-S.aur99	-	-	√	-	-	-	√	-	-
72	BoAISRF-S.aur100	-	-	√	-	-	-	-	-	-
73	BoAISRF-S.aur101	-	-	-	-	-	√	-	-	-
74	BoAISRF-S.aur102	-	-	-	√	-	-	-	√	-

75	BoAISRF-S.aur103	-	-	√	-	-	-	√	-	-
76	BoAISRF-S.aur104	-	-	-	√	-	-	-	√	-
77	BoAISRF-S.aur105	-	-	-	-	-	√	-	-	-
78	BoAISRF-S.aur106	-	-	-	-	-	-	-	-	-
79	BoAISRF-S.aur107	-	-	-	-	-	-	-	-	-
80	BoAISRF-S.aur108	-	-	-	-	-	-	-	-	-
81	BoAISRF-S.aur109	-	-	√	-	-	-	√	-	-
82	BoAISRF-S.aur110	-	-	√	-	-	-	√	-	-
83	BoAISRF-S.aur111	-	-	-	√	-	-	-	√	-
84	BoAISRF-S.aur112	-	-	-	-	-	-	-	-	-
85	BoAISRF-S.aur113	-	-	-	-	-	-	-	-	-
86	BoAISRF-S.aur114	-	-	-	-	-	√	-	-	-
87	BoAISRF-S.aur115	-	-	-	-	-	√	-	-	-
88	BoAISRF-S.aur116	-	-	-	√	-	-	-	√	-
89	BoAISRF-S.aur117	-	-	-	√	-	-	-	√	-
90	BoAISRF-S.aur118	-	-	-	√	-	-	-	√	-
91	BoAISRF-S.aur119	-	-	-	√	-	-	-	√	-
92	BoAISRF-S.aur120	-	-	√	-	-	-	√	-	-
93	BoAISRF-S.aur121	-	-	√	-	-	-	√	-	-
94	BoAISRF-S.aur122	-	-	-	-	-	-	-	-	-
95	BoAISRF-S.aur123	-	-	-	-	-	-	-	-	√

96	BoAISRF-S.aur124	-	-	-	√	-	-	-	√	-
97	BoAISRF-S.aur125	-	-	√	-	-	-	√	-	-
98	BoAISRF-S.aur126	-	-	-	√	-	-	-	√	-
99	BoAISRF-S.aur127	-	-	-	√	-	-	-	√	-
100	BoAISRF-S.aur128	-	-	-	-	-	-	-	-	-
101	BoAISRF-S.aur129	-	-	-	-	-	-	-	-	-
102	BoAISRF-S.aur130	-	-	-	-	-	√	-	-	-
103	BoAISRF-S.aur131	-	-	-	-	-	-	-	-	-
104	BoAISRF-S.aur132	-	-	-	-	-	-	-	-	-
105	BoAISRF-S.aur133	-	-	-	√	-	-	-	√	-
106	BoAISRF-S.aur134	-	-	-	-	-	-	-	-	-
107	BoAISRF-S.aur135	-	-	-	-	-	-	-	-	-
108	BoAISRF-S.aur136	-	-	-	√	-	-	-	√	-
109	BoAISRF-S.aur137	-	-	-	-	-	-	-	-	-
110	BoAISRF-S.aur138	-	-	√	-	-	-	√	-	-
111	BoAISRF-S.aur139	-	-	-	-	-	-	-	-	-
112	BoAISRF-S.aur140	-	-	√	-	-	-	-	-	-
113	BoAISRF-S.aur141	-	-	-	√	-	-	-	√	-
114	BoAISRF-S.aur142	-	-	-	-	-	-	-	-	√
115	BoAISRF-S.aur143	-	-	-	-	-	-	-	-	-
116	BoAISRF-S.aur144	-	-	-	-	-	-	-	-	-
117	BoAISRF-S.aur145	-	-	√	-	-	-	√	-	-
118	BoAISRF-S.aur146	-	-	√	-	-	-	√	-	-

119	BoAISRF-S.aur147	-	-	-	-	-	-	-	-	√
120	BoAISRF-S.aur148	-	-	-	√	-	-	-	-	-
121	BoAISRF-S.aur149	-	-	-	√	-	-	-	√	-
122	BoAISRF-S.aur150	-	-	√	-	-	-	√	-	-
123	BoAISRF-S.aur151	-	-	-	√	-	-	-	√	-
124	BoAISRF-S.aur152	-	-	-	√	-	-	-	√	-
125	BoAISRF-S.aur153	-	-	√	-	-	-	√	-	-
126	BoAISRF-S.aur154	-	-	-	-	-	-	-	-	-
127	BoAISRF-S.aur155	-	-	√	-	-	-	√	-	-
128	BoAISRF-S.aur156	-	-	-	√	-	-	-	√	-
129	BoAISRF-S.aur157	-	-	-	√	-	-	-	√	-
130	BoAISRF-S.aur158	-	-	-	√	-	-	-	√	-
131	BoAISRF-S.aur159	-	-	√	-	-	-	√	-	-
132	BoAISRF-S.aur160	-	-	√	-	-	-	√	-	-
133	BoAISRF-S.aur161	-	-	√	-	-	-	√	-	-
134	BoAISRF-S.aur162	-	-	-	√	-	-	-	√	-
135	BoAISRF-S.aur163	-	-	√	-	-	-	√	-	-
136	BoAISRF-S.aur165	-	-	-	-	-	√	-	-	-
137	BoAISRF-S.aur166	-	-	√	-	-	-	√	-	-
138	BoAISRF-S.aur167	-	-	√	-	-	-	√	-	-
139	BoAISRF-S.aur168	-	-	-	-	-	-	-	-	-
140	BoAISRF-S.aur169	-	-	-	-	-	-	-	-	-
141	BoAISRF-S.aur170	-	-	-	-	-	-	-	-	-
142	BoAISRF-S.aur171	-	-	-	-	-	-	-	-	-
143	BoAISRF-S.aur172	-	-	-	-	-	-	-	-	-
144	BoAISRF-S.aur173	-	-	-	-	-	-	-	-	-
145	BoAISRF-S.aur174	-	-	√	-	-	-	√	-	-

146	BoAISRF-S.aur175	-	-	-	-	-	-	-	-	-
147	BoAISRF-S.aur176	-	-	√	-	-	-	√	-	-
148	BoAISRF-S.aur177	-	-	-	-	-	√	-	-	-
149	BoAISRF-S.aur178	-	-	-	-	-	-	-	-	√
150	BoAISRF-S.aur179	-	-	√	-	-	-	-	-	-
151	BoAISRF-S.aur180	-	-	-	-	-	-	-	-	-
152	BoAISRF-S.aur181	-	-	√	-	-	-	√	-	-
153	BoAISRF-S.aur182	-	-	√	-	-	-	√	-	-
154	BoAISRF-S.aur183	-	-	-	√	-	-	-	√	-

B. 4: Chapter 6

Table 1A: Total viable counts of *S. aureus* recovered from mammary glands 48 h post-harvest

Group	Raw value	x80x10^4	log10
117.1	67	53600000	7.729165
117.2	67	53600000	7.729165
117.3	65	52000000	7.716003
117.4	66	52800000	7.722634
48.1	104	83200000	7.920123
48.2	104	83200000	7.920123
48.3	103	82400000	7.915927
48.4	104	83200000	7.920123
51.1	150	1.2E+08	8.079181
51.2	153	1.22E+08	8.087781
51.3	150	1.2E+08	8.079181
51.4	149	1.19E+08	8.076276
104.1	43	34400000	7.536558
104.2	44	35200000	7.546543
104.3	40	32000000	7.50515
104.4	41	32800000	7.515874

Table 1B: Total viable counts of *S. aureus* recovered from mammary glands 48 h post-harvest

Group	Average	Standard deviation	Standard error
117	7.724242	0.006296271	0.003148136
48	7.919074	0.002098057	0.001049029
51	8.080605	0.004976383	0.002488191
104	7.526031	0.018892152	0.009446076

Table 1.C: Statistical analysis of Total viable counts of *S. aureus* recovered from mammary glands 48 h post-harvest

117	48	51	104
53600000	83200000	120000000	34400000
53600000	83200000	122400000	35200000
52000000	82400000	120000000	32000000
52800000	83200000	119200000	32800000

```
oneway DV Group, bonferroni tabulate

        |          Summary of DV
    Group |      Mean   Std. Dev.      Freq.
------------+------------------------------------
        1 |    53000000   765941.69         4
        2 |    83000000     400000         4
        3 |   1.204e+08   1385640.6         4
        4 |    33600000   1460593.5         4
------------+------------------------------------
    Total |    72500000    33869239        16

                Analysis of Variance
   Source          SS       df      MS         F     Prob > F
------------------------------------------------------------------------
Between groups     1.7192e+16     3   5.7308e+15   4775.69    0.0000
Within groups      1.4400e+13    12   1.2000e+12
------------------------------------------------------------------------
   Total         1.7207e+16    15   1.1471e+15

Bartlett's test for equal variances:  chi2(3) =   4.4389  Prob>chi2 = 0.218

                   Comparison of DV by Group
                         (Bonferroni)
Row Mean-|
Col Mean |        1         2         3
---------+---------------------------------
      2 |    3.0e+07
        |      0.000
        |
      3 |    6.7e+07    3.7e+07
        |      0.000      0.000
        |
      4 |   -1.9e+07   -4.9e+07   -8.7e+07
        |      0.000      0.000      0.000
```

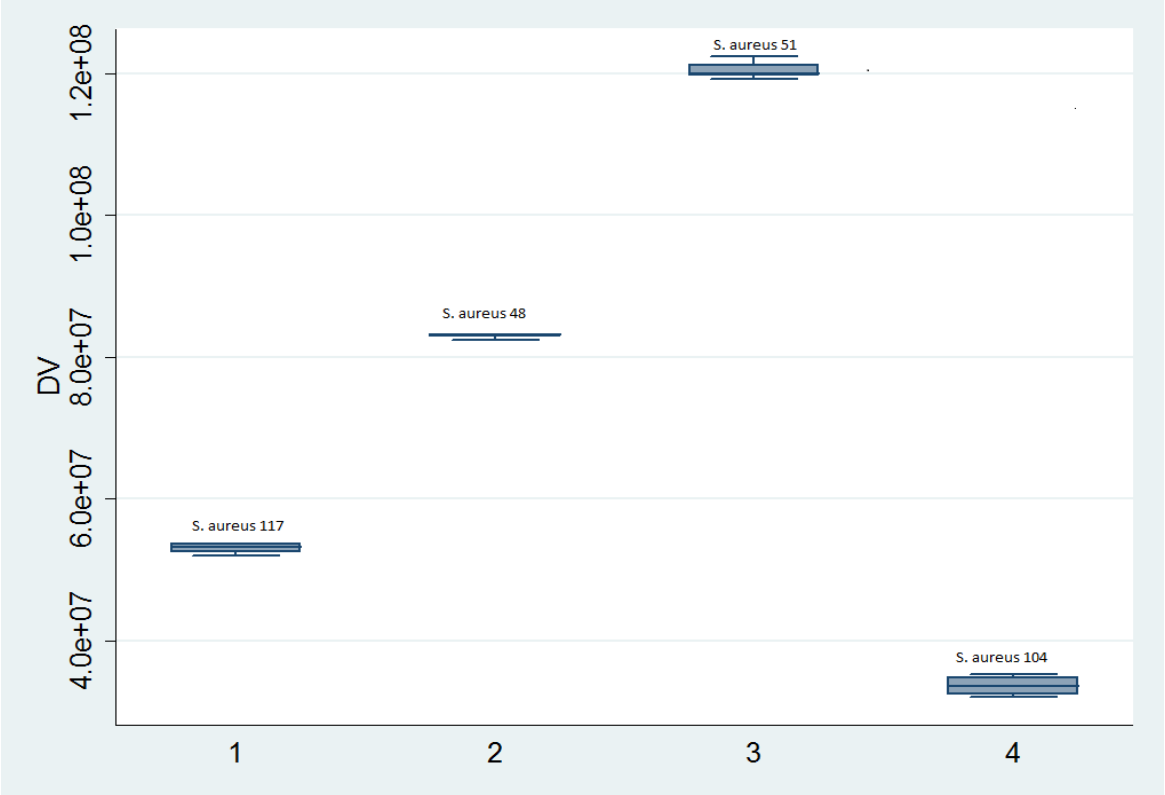

Table 2 : Flow cytometry data analysis

IL10	48	51	117	104
	12.15587	3.842962	4.127976	6.550594
	11.01581	5.054271	3.629202	9.020714
	14.49217	3.842962	4.983017	7.76114
	12.60332	7.619396	4.49217	5.07726
avg	12.70377	5.505543	4.36813	7.286371
SD	1.44716	1.780386	0.572347	1.685185
SE	0.72358	0.890193	0.286173	0.842593
IL12	13.25523	1.468591	1.094724	7.017558
	8.69012	2.767286	0.937307	8.57405
	12.80664	0.976661	1.252142	8.69012
	14.40049	3.495342	2.452451	10.71687
avg	12.28812	2.17697	1.434156	8.74965
SD	2.490775	1.158896	0.690924	1.517067
SE	1.245388	0.579448	0.345462	0.758533
IL17A	2.345293	0.147321	1.112028	2.37838
	2.583525	0.150469	1.138691	1.588685
	2.998225	0.067911	1.233543	1.571038

	2.645288	0.068852	1.165161	1.14972
avg	2.643083	0.108638	1.162356	1.671956
SD	0.269788	0.046504	0.052181	0.512797
SE	0.134894	0.023252	0.02609	0.256399
IFN-γ	8.114035	12.58865	7.640868	8.04124
	8.525782	11.09635	7.888825	8.380191
	4.144436	7.952521	8.40294	14.71563
	7.548	7.75916	7.117654	13.01632
avg	7.083063	9.84917	7.762572	11.03835
SD	1.99967	2.38227	0.534398	3.34082
SE	0.999835	1.191135	0.267199	1.67041

B. 5: Chapter 7

Table 1A: Table 1A: Total viable counts of *S. aureus* recovered from mammary glands 48 h post-harvest

Group	Raw value	x80x10^4	log10
83.1	210	1.68E+08	8.225309
83.2	212	1.7E+08	8.229426
83.3	209	1.67E+08	8.223236
83.4	209	1.67E+08	8.223236
87.1	104	83200000	7.920123
87.2	102	81600000	7.91169
87.3	100	80000000	7.90309
87.4	102	81600000	7.91169

Table 1B: Table 1A: Total viable counts of *S. aureus* recovered from mammary glands 48 h post-harvest

Group	Average	Standard deviation	Standard error
83	8.225302	0.002917798	0.001458899
87	7.911648	0.006953999	0.003476999

Table 2: Flow cytometry data analysis

	IL-10	IL-12	IL-17	IFNgama	
	68.84988	2.67353	33.71914	57.11185	
	65.99973	2.19311	28.13833	64.80309	
	64.47934	3.08212	27.03099	58.01622	s.aur83
	67.7619	4.164366	25.51337	58.05489	
avg	66.77271	3.028282	28.60046	59.49651	
SD	1.927881	0.840029	3.578073	3.564451	
SE	0.963941	0.420015	1.789036	1.782226	
	12.34588	1.094724	18.52964	14.95448	
	12.66485	0.937307	19.20904	8.25735	
	11.60959	1.252142	18.20979	13.6146	
	14.81676	2.452451	16.83775	13.95583	s aur87
avg	12.85927	1.434156	18.19656	12.69557	
SD	1.37778	0.690924	0.997095	3.012936	
SE	0.68889	0.345462	0.498548	1.506468	

Table 3: Bacterial load of mammary glands in anti-inflammatory cytokine treated mice

(a) IL-6 control mice group

Mouse	CFU	Log
1	216000000	8.334454
2	208000000	8.318063
3	176000000	8.245513
4	160000000	8.20412
5	232000000	8.365488
6	200000000	8.30103
	avg	8.294778
	SD	0.059668
	SE	0.024359

(b) Il-6 test mice group

Mouse	CFU	Log
1	50400000	7.702431
2	45600000	7.658965
3	49600000	7.695482
4	37600000	7.575188
5	44000000	7.643453
6	34400000	7.536558
	avg	7.635346
	SD	0.06651
	SE	0.027153

(c) IL-6R control mice group

Mouse	CFU	Log
1	160000000	8.20412
2	168000000	8.225309
3	200000000	8.30103
4	168000000	8.225309
5	256000000	8.40824
6	112000000	8.049218
	avg	8.235538
	SD	0.118344
	SE	0.048314

(d) IL-6R test mice group

Mouse	CFU	Log
1	48800000	7.68842
2	41600000	7.619093
3	40000000	7.60206
4	32000000	7.50515
5	44000000	7.643453
6	25600000	7.40824
	avg	7.577736
	SD	0.102829
	SE	0.04198

B.6: Chapter 8

Table 1: Humoral immune response of Planktonic *S. aureus* vaccine by s/c route

Mouse serum	IgG	IgG1
1	60000	80000
2	60000	80000
3	75000	58000
4	60000	20000
5	32000	20000
6	32000	40000
avg	53166.67	49666.67
SD	17394.44	27434.77
SE	7101.252	11200.2

Table 2: Humoral immune response Planktonic *S. aureus* vaccine by i/mam route

Mouse serum	IgG	IgG1
1	16000	12500
2	2500	10000
3	34000	12500
4	20000	15000
5	10000	20000
6	10000	20000
avg	15416.67	15000
SD	10883.09	4183.3
SE	4443.003	1707.825

Table 3: Humoral immune response Biofilm *S. aureus* vaccine by s/c route

Mouse serum	IgG	IgG1	IgG2a
1	26500	20000	6000
2	28000	21000	14000
3	25000	13000	14000
4	16000	20000	4500
5	15000	20000	4500
6	16000	7000	6000
avg	21083.33	16833.33	8166.667
SD	6020.105	5636.193	4568.005
SE	2457.698	2300.966	1864.88

Table 4: Humoral immune response Biofilm *S. aureus* vaccine by i/mam route

Mouse serum	IgG	IgG1	IgG2a
1	40000	14000	28000
2	35000	6000	35000
3	40000	8000	35000
4	37500	12000	29500
5	40000	12000	30000
6	45000	12000	30000
avg	39583.33	10666.67	31250
SD	3322.9	3011.091	2995.83
SE	1356.568	1229.273	1223.043

Table 5: Concentration of IFN-γ produced by killed planktonic *S. aureus* stimulated splenocytes of mice immunized with planktonic *S. aureus* vaccine using different routes

Mouse splenocyte sample	s/c route	i/mam route	ConA
1	77.6	57.2	6100
2	0	0	6200
3	77.6	0	6150
4	56.4	57.2	6200
5	0	0	6200
6	77.6	0	6350
avg	48.2	19.06667	6200
SD	38.22774	29.53795	83.666
SE	15.60641	12.05882	34.1565

Table 6: Concentration of IFN-γ produced by killed biofilm *S. aureus* stimulated splenocytes of mice immunized with biofilm *S. aureus* vaccine using different routes

Mouse splenocyte sample	s/c	i/mam	ConA
1	0	5246.8	6150
2	616.4	2830	6200
3	808.8	3621.2	6000
4	0	4540.8	6350
5	616.4	4327.2	6100
6	723.6	4990	6200
avg	460.8667	4259.333	6166.667
SD	364.2153	899.2221	116.9045
SE	148.6903	367.1059	47.72607

Table 7: Detection of bacterial load of the mammary glands of mice vaccinated with *S. aureus* planktonic and biofilm vaccines

(a) Planktonic control mice (i/mam route)

Mouse	CFU	Log value
1	40000000	7.60206
2	4000000	6.60206
3	200000	5.30103
4	1E+08	8
5	400000	5.60206
6	1E+08	8
	avg	6.851202
	SD	1.202391
	SE	0.490874

(b) Planktonic control mice (s/c route)

Mouse	CFU	Log value
1	40000000	7.60206
2	40000000	7.60206
3	40000000	7.60206
4	60000000	7.778151
5	80000000	7.90309
6	40000000	7.60206
	avg	7.68158
	SD	0.129373
	SE	0.052816

(c) Planktonic test mice (s/c route)

Mouse	CFU	Log value
1	4000000	6.60206
2	4000000	6.60206
3	4000000	6.60206
4	2000000	6.30103
5	600000	5.778151
6	40000000	7.60206
	avg	6.581237
	SD	0.594448
	SE	0.242682

(d) Planktonic test mice (i/mam route)

Mouse	CFU	Log value
1	140000000	8.146128
2	12000000	7.079181
3	4000000	6.60206
4	4000000	6.60206
5	600000	5.778151
6	400000	5.60206
	avg	6.63494
	SD	0.925649
	SE	0.377895

(e) Biofilm control mice (s/c route)

Mouse	CFU	Log value
1	20000000	7.30103
2	4000000	6.60206
3	200000	5.30103
4	100000000	8
5	400000	5.60206
6	100000000	8
	avg	6.80103
	SD	1.170656
	SE	0.477918

(f) Biofilm control mice (i/mam route)

Mouse	CFU	Log value
1	40000000	7.60206
2	20000000	7.30103
3	40000000	7.60206
4	60000000	7.778151
5	80000000	7.90309
6	40000000	7.60206
	avg	7.631409
	SD	0.203508
	SE	0.083082

(g) Biofilm test mice (s/c route)

(

Mouse	CFU	Log value
1	500000	5.69897
2	300000	5.477121
3	340000	5.531479
4	240000	5.380211
5	400000	5.60206
6	440000	5.643453
	avg	5.555549
	SD	0.116562
	SE	0.047586

(h) Biofilm test mice (i/mam route)

Mouse	CFU	Log value
1	354000	5.549003
2	380000	5.579784
3	400000	5.60206
4	330000	5.518514
5	358000	5.553883
6	400000	5.60206
	avg	5.567551
	SD	0.033066
	SE	0.013499

B.7: Chapter 9

Table 1: Detection of antigen-specific antibody in sera samples of mice vaccinated with Protein A

Protein A s/c route

	IgG	IgG1	IgG2a
	220	200	80
	400	240	100
	650	480	200
	1200	500	100
	300	220	100
	420	200	120
Avg	531.6667	306.6667	116.6667
SD	358.1294	142.9219	42.73952
SE	146.2057	58.34762	17.44834

ProteinA i/mam

	IgG	IgG1	IgG2a
	280	80	120
	220	40	120
	220	40	140
	255	40	140
	240	40	120
	240	40	120
Avg	242.5	46.66667	126.6667
SD	22.74863	16.32993	10.32796
SE	9.287088	6.666667	4.21637

Table 2: Concentration of IFN-γ produced by splenocytes of Protein A stimulated mice immunized by Protein A using different routes.

	IFN gama level			
	ProteinA s/c	ProteinA i/mam	ProteinA PBS s/c	PBS i/mam
	183.94	163.62	31.56	23.1
	123.52	213.34	33.69	21
	143.3	179.125	30.489	22.2
	166.29	174.845	25.14	22.12
	160.95	120.8473	25.67	20.7
	177.52	266.2756	26.21	23
Avg	159.0233	186.3422	28.79317	22.02
SD	22.48055	49.21053	3.585603	0.995188
SE	9.177647	20.09012	1.463816	0.406284

Table 3: Detection of bacterial load of the mammary glands of mice immunized with Protein A

(a) Protein A s/c route

CFU	Log value
80000000	7.90309
100000000	8
80000000	7.90309
340000000	8.531479
460000000	8.662758
120000000	8.079181
196666667	8.293731
AVG	8.179933
SD	0.33242
SE	0.13571

(b) Protein A i/mam route

CFU	Log value
60000000	7.778151
1300000	6.113943
6000000	6.778151
60000000	7.778151
60000000	7.778151
60000000	7.778151
Avg	7.334117
SD	0.719248
SE	0.293632

(c) Protein A control mice (s/c route)

CFU	Log
2000000000	9.30103
1000000000	9
1200000000	9.079181
1000000000	9
1000000000	9
700000000	8.845098
Avg	9.037552
SD	0.149948
SE	0.061216

(d) Protein A control mice (i/mam route)

CFU	Log value
1700000000	9.230449
1000000000	9
1300000000	9.113943
1000000000	9
1000000000	9
900000000	8.954243
Avg	9.049772
SD	0.103266
SE	0.042158