School of Biomedical Sciences

Characterization and identification of novel biofilm forming antigens of *Staphylococcus aureus* of human origin

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This thesis is dedicated to my parents, Gurbakhsh Singh and MaryAnn Babra and my husband, Onkar Singh Waryah.

DECLARATION

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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PUBLICATIONS

Published Papers

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ABSTRACT

Treatment of infections caused by *Staphylococcus aureus* remains one of the biggest challenges owing to the versatility of the nosocomial pathogen continually developing increased resistance to the traditional as well the novel antibiotics. This opportunistic pathogen is part of the human natural flora and resides in nasal passages and on skin. In addition, *S. aureus* is one of the major contributors in community and hospital acquired infections due to development of resistance to multiple antibiotics, including vancomycin, a last resort antibiotic. It causes infections ranging from skin and soft tissue infections to invasive disease syndromes including pneumonia, septicaemia, endocarditis and osteomyelitis.

A major reason for the difficulty encountered in winning the battle with *S. aureus* is its ability to adapt to the host environment and overcoming the host defenses by producing over 50+ virulence factors making it a truly versatile pathogen. Human *S. aureus* can express all or a mixture of these factors that may facilitate the bacterium's ability to attach itself to a variety of cell surface structures, that in turn allow the bacteria to establish an infection. These include, but are not limited to, expression of exotoxins, extracellular capsular polysaccharide, surface poly-N-acetyl-(1,6)- β-D-glucosamine (PNAG), teichoic acid and numerous surface-associated protein adhesins collectively known as microbial surface component recognizing adhesive matrix molecules or MSCRAMM. A general feature of *S. aureus,* as with many other pathogens, is the formation of a self-produced matrix referred to as a biofilm, which protects the pathogen from host defenses and antimicrobial agents. Given the importance of *S. aureus* as a nosocomial and community pathogen, and its ability to develop resistance to antimicrobials and antibiotics, exploration of strategies to prevent biofilm formation is of utmost importance. Definitive information on the nature of the virulence factors, known and unknown, participating in the formation of biofilm is sparse, hence the aim of this investigation.

In this thesis, results from this study are assembled in the form of a series of scientific publications which have either been published or submitted to scientific journals for peer review. The potential roles of biofilm formation that may assist the *S. aureus* in the establishment infection in the host have been analyzed using novel *in vitro* approaches and the outcomes embodied this thesis listed below are presented.

1. Biofilm formation by *S. aureus* was shown to be associated with development of persistent antibiotic resistance.
2. The lack of association between capsular polysaccharide and biofilm formation was demonstrated.
3. Substantial, albeit not absolute association of the possession of PNAG and biofilm formation was demonstrated.
4. The presence of additional capsular types different from the currently accepted 4 types was demonstrated.
5. The distribution of key virulence factors of *S. aureus* in Western Australian isolates was demonstrated to be diverse with respect to a wide array of virulence factors including MSCRAMM and exotoxins.
6. A treatment strategy involving the use of biofilm degrading/dispersing enzymes, DNAse I and dispersin B, for infections caused by *S. aureus* in combination with a model antibiotic tobramycin, for enhancing the antimicrobial efficacy was evaluated *in vitro*. It was demonstrated for the first time that combination of 2 biofilm-degrading enzymes resulted in a 129-fold reduction in the efficacy of the model antibiotic, tobramycin. In contrast, the use of these enzymes as singular molecules was more effective in enhancing the efficacy of tobramycin.
7. Preliminary immunoproteomic analysis of the biofilm of *S. aureus* grown under the stress of iron deprivation revealed the identification of a novel, previously unknown biofilm-associated antigen, Fructose 1,6-bisphosphate alsolase class 1 (FBA-1), a conserved enzyme with little homology with the human equivalent.
8. FBA-1 was found to be involved in biofilm formation as determined by the novel peg-based antibody-mediated biofilm inhibition technique to the same

extent as the biofilm-associated immunogenic and protective manganese transporter protein C.

9. Immunogenicity and protective potential of FBA-1, determined using the acute murine bacteraemia model but found to be significantly inferior to the standard MntC molecule.
10. Future directions for the development of a biofilm-antigens based vaccine against infections caused by *S. aureus*, including strategies for the selection of vaccine candidates for the development of cocktail and/or conjugate vaccines.

STATEMENT OF CONTRIBUTION

I hereby declare that work published and presented in this thesis was designed, written, experimentally conducted and interpreted by myself, Charlene Waryah.

Chapters 1, 2 and **10** – Associate Professor TK Mukkur reviewed these chapters and provided critical comments.

Chapter 3 – Dr Jully Gogoi-Tiwari, Associate Professor TK Mukkur provided significant contribution to the design of the study. Dr Paul Costantino, Dr Raju Sunagar, Dr Nag Hedge and Dr Shrikrishna Isloor provided critical revision and interpretation of data.

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Chapter 1 - Literature Review

1.1 *Staphylococcus aureus* – the pathogen

1.1.1 Classification and Colony Morphology

Staphylococcus was first discovered in 1880 by a Scottish surgeon named Sir Alexander Ogston from a pus abscess (1984). It was later named *Staphylococcus aureus* (*S. aureus*) by Friedrich Julius Rosenbach who is credited for differentiating *S. aureus* from *Staphylococcus epidermidis*, formerly known as *Staphylococcus albus* (Licitra, 2013).

Staphylococcus aureus is facultative anaerobic, gram-positive cocci that grow in large, golden-yellow colonies on blood agar, often observed with beta haemolysis (Cowan et al., 1993). The Greek name is derived from its characteristic “grape-like” clusters (*Staphyle*) when viewed under the microscope and golden pigment (*aureus*) when grown on blood agar, which also gives rise to its commonly used name of “Golden Staph” (Cowan et al., 1993, Liu et al., 2005).

1.1.2 Diseases caused by *Staphylococcus aureus*

Staphylococcus aureus is part of the normal flora and has the ability to asymptomatically colonize healthy individuals (O'Riordan and Lee, 2004). It is estimated 20-30% of individuals carry this bacterium as a part of the normal microflora (Huda et al., 2011). The presence of bacteria on the host skin does not indicate an infection but is a significant and common source of transfer to other subjects (Krismer et al., 2014). Subject to breaches in the integrity of physical and physiological barriers of the innate immune defences, *S. aureus* can become an opportunistic pathogen (O'Riordan and Lee, 2004). Diseases caused by *S. aureus* are alarmingly large and range from acute to chronic infections due to a variety of virulence factors (Kropec et al., 2005). Infections cause by *S. aureus* can be classified into superficial infections, toxin mediated infections, and life threatening invasive infections such as sepsis (Blaiotta et al., 2006).

Superficial lesions range from mild boils and pimples, stys, abscesses, carbuncles, wound infections and the like. Upon penetration of the skin barrier, the threat can increase to muscular and skeletal infections such as osteomyelitis and septic arthritis. These can further lead to the serious conditions of bacteraemia, pneumonia, endocarditis and septicaemia (Cramton et al., 1999, Ando et al., 2004, Anderson et al., 2012b, Dinges et al., 2000).

Toxin mediated infections caused by *S. aureus* are due to its ability to produce a wide range of toxins including superantigens, exfoliative toxins and cytotoxic toxins (Blaiotta et al., 2006). The toxin-associated diseases include food poisoning, toxic shock syndrome and scaled skin syndrome (Dinges et al., 2000).

In a hospital setting, the infections range further to ventilator associated pneumonia, device related infections such as endotracheal tubes, intravascular and urinary catheters, prosthetic implants and arterial stents (Anderson et al., 2012b). In a community setting, cases of infection are generally skin and soft-tissue related infections (Shukla et al., 2010).

1.1.3 Burden of infection and antibiotic treatment

Staphylococcus aureus cause an increased burden in post-surgery intensive care units (ICUs), especially in immune-compromised patients including neonates and elderly patients in Australia/New Zealand and USA (James et al., 2008, Turnidge et al., 2009) . In USA alone, mortality due to pure *S. aureus* infections exceeds patient mortality caused by the influenza, hepatitis virus and HIV/AIDS (Falugi et al., 2013). *Staphylococcus aureus* antibiotic resistance rates have steadily increase from 27% to 54.1% between 1994 and 2004, $p<.001$ (Klevens et al., 2008). In the USA alone, during a 4 yr period, deaths recorded from bloodstream *S. aureus* infections were 3 fold higher than ceftazidime-resistant pneumonia related *Pseudomonas aeruginosa* infections and a staggering 9 folder higher than ciprofloxacin-resistant *Escherichia coli* (Klevens et al., 2008). More recently, a study in 2009 in Australia and New Zealand found that out of 1994 episodes of bloodstream *S. aureus* infections had a

30-day all-cause mortality of 20.6% and infection-only mortality rate of 13% (Turnidge et al., 2009).

1.1.3.1 Emergence of Antibiotic resistance

Antibiotic resistance largely depends on bacterial environment influenced by nutrients available and the introduction of antibiotics (Schentag et al., 1998).

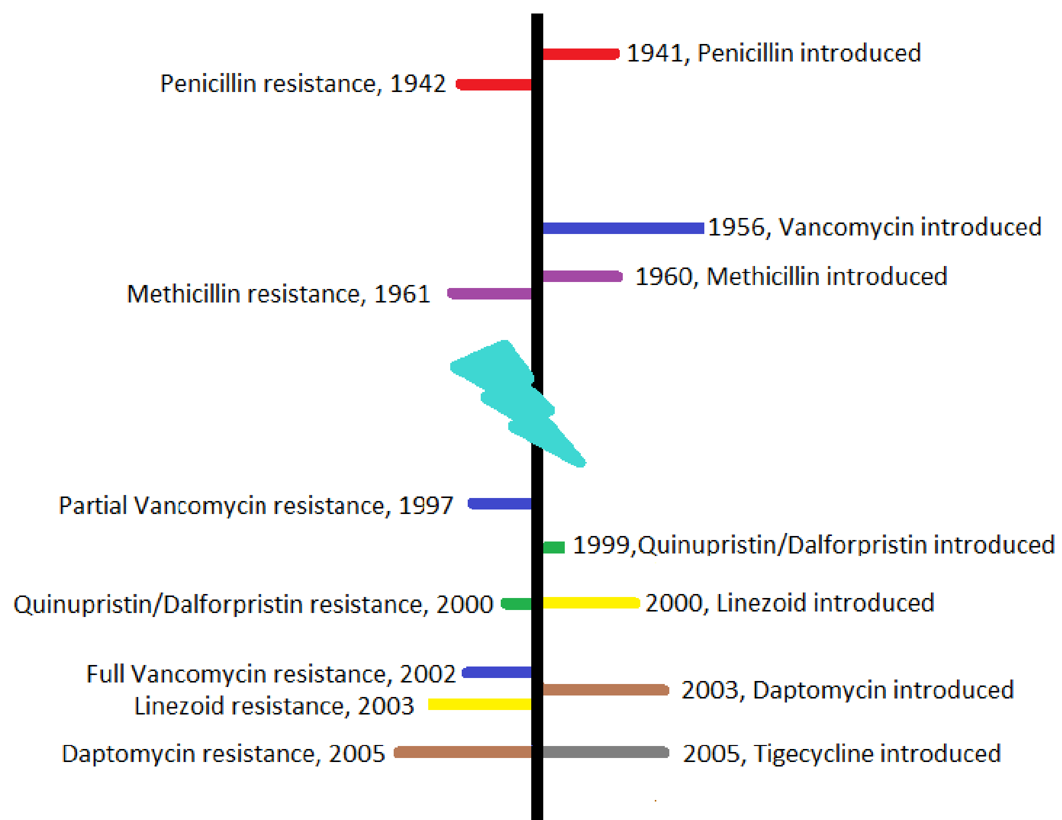
Mortality rate of *S. aureus* infection in the pre-antibiotic era was over 80%. However, the introduction of penicillin in the early 1940 dramatically improved treatment of infection (Davies and Davies, 2010). Penicillin resistance was first seen in early 1942 and by 1960s; more than 80% of nosocomial and community infections were resistant to the "wonder drug" penicillin. Resistance to penicillin is mediated by the *blaz* gene encoding the production of the enzyme β-lactamase which cleaves the β-lactam ring rendering the penicillin molecule ineffective (Lowy, 2003).

This prompted the development of semi-synthetic penicillins following the emergence of alarmingly large number of penicillinase producing bacteria in the 1960s (Levy, 2002). Currently in Australia, more than 80% of *S. aureus* strains produce penicillinase (Rayner and Munckhof, 2005). Methicillin, developed in 1961, was one of the first semisynthetic penicillinase-resistant antibiotics (Enright et al., 2002). Similar to penicillin, methicillin resistance was soon reported following its introduction and remains a challenge in the treatment of human infections.

Methicillin-resistant *S. aureus*, MRSA, strains all carry a mobile staphylococcal cassette chromosome that contains the *mecA* gene, the gene carrying methicillin resistance (Tsubakishita et al., 2010). This genetic element can be horizontally transferred from one strain to other strains of *S. aureus* and using data from epidemic MRSA outbreaks, only a limited number of clones are considered to be responsible (Lowy, 2003). The *mecA* gene encodes a penicillin-binding protein 2a, or PBP2a which is a membrane-bound transpeptidase that catalyses the cross-linkage of bacterial peptidoglycan (Hanssen and Ericson Sollid, 2006). The enzyme has a low

affinity for β-lactams therefore increases survival of bacteria during high concentrations of the antibiotic (Shukla et al., 2010).

A timeline outlining the development of resistance by *S. aureus* to important antibiotics illustrated in the following diagram.



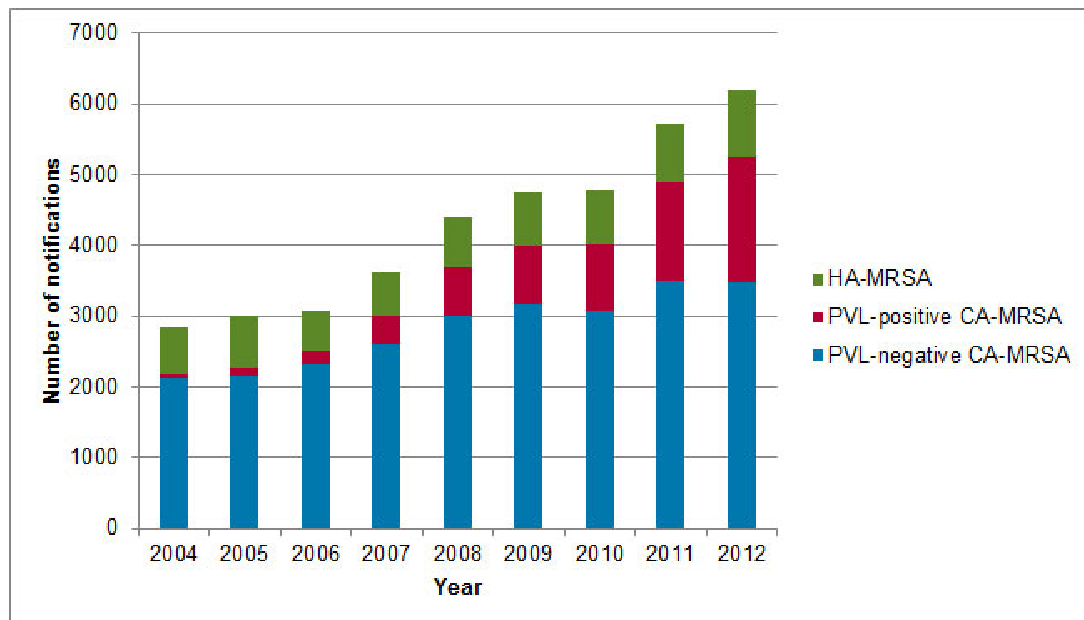
Timeline 1: Chronology of the development of antibiotic resistance in *S. aureus* (Lubelchek and Weinstein, 2008). Denotes time break.

With the introduction of antibiotics for treatment, the emergence of antibiotic resistance soon followed. *Staphylococcus aureus* is continuing to show a consistent and disturbingly high ability to develop resistance to antibiotics and antimicrobials, for example, MRSA (Hoen, 2004). The spread of infection was predominant in hospitals during the 1960s until late 1980s (hospital acquired MRSA, or HA-MRSA) but was soon discovered to be spreading in the community (community acquired MRSA, or CA-MRSA) (Dukic et al., 2013). The emergence and distribution of both HA-MRSA and CA-MRSA, has been documented worldwide in every continent now (von Eiff et al., 2007) including Western Australia (Fig 1).

Emergence of CA-MRSA has been observed in households, athletic and sporting facilities, schools, jails and nursing homes and a national survey conducted in the US found that MRSA caused more than half of *S. aureus* infections in ICUs (Dukic et al., 2013).

Improved hospital procedures such as hand washing, adequate sterilization and general awareness have started a decline in the incidence of infection. However, this has not stopped the rise of antibiotic resistance (Collignon and Cruickshank, 2009). Due to the range of infections and frequency, the increase usage of antibiotics makes the treatment of *S. aureus* difficult and an uphill battle in eradicating infections caused by this pathogen (Schentag et al., 1998).

Fig 1: Increasing incidence of HA-MRSA and CA-MRSA in Western Australia



Source: Disease Watch Vol 17, Issues 3, Department of Health, Government of Western Australia (WAtch, 2013). A steady increase over time can be observed in CA-MRSA either with or without expression of the Panton-Valentine leukocidin, a toxin produced by some *S. aureus* strains associated with CA-MRSA.

1.1.3.2 Current Antibiotic Treatment for Staphylococcal Infections

The first line of antibiotics for treatment of staphylococcal infections with methicillin-sensitive *S. aureus* (MSSA) still includes semi-synthetic penicillin such as oxacillin and oral dicloxacillin (Eliopoulos, 2004). Should the patient be allergic to penicillin, treatment with of first generation cephalosporins is administered (Stryjewski et al., 2007). For more serious infections with MRSA, treatment with the last resort antibiotic, Vancomycin which is non-selection, is delivered intravenously (Miller and Rudoy, 2000).

1.2 Key Virulence Factors

The reason why *S. aureus* causes such a wide range of infection types is due to the number of virulence factors it produces, which vary in expression and production from strain to strain. An impressive 50 plus virulence factors are produced by *S. aureus* isolates which include a variety of toxins, host cell adhesion factors, biofilm formation and a production of capsule (Broughan et al., 2011). A summary of the major virulence factors produced by *S. aureus* are summarized in Table 1.

Table 1: Summary of the major virulence factors, their activity and the corresponding genes encoding their production.

Activity	Virulence Factor	Encoding genes	Associated with and/or action	References
Immune evasion	Staphylococcal protein A	*Spa*	Prevents phagocytosis and acts as an immunological disguise	(Stutz et al., 2011)
	Production of capsule Types 1, 2, 5 and 8	*cap1, cap2, cap5* and *cap8*	Renders bacteria resistant to phagocytosis	(O'Riordan and Lee, 2004)
Toxins	Haemolytic toxins Alpha, Beta, Gamma, Delta	*Hla, hlb, hlg, hld*	Destruction of red blood cells, associated with invasive disease	(Wehrhahn et al., 2012)
	Leukocidin Panton Valentine leucocidin two components LukS-PV and LukF-PV	*lukF, lukS*	Destruction of leukocytes, associated with skin and soft-tissue infection	(Daskalaki et al., 2010)
	Pyrogenic toxin superantigens Staphylococcal enterotoxins (A-E, G-J)	*sea-see, seg-sej*	Food poisoning	(Pinchuk et al., 2010)
	Toxic shock syndrome toxin-1	*tst*	Toxic Shock Syndrome	(Hu et al., 2003)
	Exfoliative toxins Exfoliative toxin A and B	*eta, etb*	Staphylococcal scalded-skin syndrome	(Bukowski et al., 2010)

Adhesins	MSCRAMM* Fibronectin binding protein: FnBPa, FnBPb	*Fnbpa, fnbpb,*	Adhesion to fibronectin, intercellular adhesion	(Brouillette et al., 2003)
	Clumping factor: ClfA, ClfB	*clfa, clfb*	Adhesion to fibrinogen, platelet aggregation	(Walsh et al., 2008)
	Collagen binding protein: Cna	*cna*	Adhesion to collagen	(Svensson et al., 2001)
	Bone sialoprotein binding protein: Bbp	*bbp*	Bone and joint infections	(Persson et al., 2009)
	Iron-regulated surface determinants: IsdA and IsdB	*isdA, isdB*	Acquisition of iron by bacteria	(Kim et al., 2010b)
Biofilm formation	PNAG Poly-N-acetyl-(1,6)-β-D-glucosamine	*icaA-D***	Production of biofilm	(Maira-Litran et al., 2005)
	Teichoic acid Wall teichoic acid (WTA)	*wta*	Linked to cell wall, provide negative charge to anchor positive charged PNAG	(Vergara-Irigaray et al., 2008)
	Lipoteichoic acid (LTA)	*lta*	Linked to cell membrane, provide negative charge to anchor positive charged PNAG	(Vergara-Irigaray et al., 2008)

* Microbial Surface Components Recognizing Adhesive Matrix Molecules

** Intercellular adhesin genes encoding production of proteins IcaA, IcaB, IcaC and IcaD

1.2.1 Toxins

Staphylococcus aureus has an alarming ability to produce variety of virulence factors which include the production of exotoxins including enterotoxins, most of which have a potential detrimental effect on host immune system (Dinges et al., 2000).

Exotoxins, which are referred as superantigens, have the ability to induce an uncontrolled T-cell dependent immune response by binding to both T-cell receptors and major histocompatibility complex class II antigens which are expressed on antigen-presenting cells (Proft and Fraser, 2003). This binding triggers a chain reaction that results in the production of inflammatory cytokines IL-1 and TNF-α leading to uncontrolled fever, toxin shock, multi-organ failure and death (Proft and Fraser, 2003, Moza et al., 2007). Superantigens are large family of toxins produced by bacteria and pathogens including *S. aureus* (Proft and Fraser, 2003). More than 60% of isolated clinical samples contain genes encoding one or more superantigens (Lin and Peterson, 2010).

The superantigenic toxins including highly virulent epidermolytic toxins, ETA and ETB, which cause staphylococcal scalded skin syndrome (SSSS) (James et al., 2008) and staphylococcal enterotoxins (SE), a major toxin group, are also classified as superantigens (Proft and Fraser, 2003, Dinges et al., 2000). Based on the degree of sequence homology, over 20 enterotoxins (SEA to SEE and SEG to SEJ) (Pinchuk et al., 2010) designated according to serological specificity (Mehrotra et al., 2000, Sharma et al., 2000) have been recognised. The potency of SEs is so high that less than 1µg of toxin is adequate to induce vomiting in humans (Proft and Fraser, 2003). The very first superantigen was isolated from *S. aureus* was in the late 1960s and later identified as SEA (Proft and Fraser, 2003). The SEs and Toxic shock syndrome toxin-1 (TSST-1) are commonly known as pyrogenic toxin superantigens (Schlievert et al., 2000, Blaiotta et al., 2006). TSST-1, commonly associated with menstruating toxic shock syndrome (TSS), is responsible for 90% menstrual and about half of non-menstrual associated TSS (Lin and Peterson, 2010).

Haemolyins are another important group of toxins categorized into alpha, beta, delta and gamma toxins (Blaiotta et al., 2006). The α-toxin is a secreted water soluble 34-kDa monomer protein encoded by the *hla* gene and carried by the majority of clinical isolates (Caiazza and O'Toole, 2003, Lin and Peterson, 2010, Bien et al., 2011, Wilke and Bubeck Wardenburg, 2010). Staphylococcal α-toxin was the first bacterial exotoxin identified as a pore forming toxin that disrupts target cell membrane resulting in loss of membrane integrity, availability of nutrients and host cell death (Bien et al., 2011). The toxin engages and targets with a variety of host cells including epithelial cells and important members of the immune system (erythrocytes, lymphocytes and macrophages). Higher concentrations of α-toxin permits nonspecific absorption of the toxin into the lipid bilayer forming Ca^{2+} permissive channels (Bien et al., 2011). This action not only disrupts cell integrity but permits an uncontrolled Ca^{2+} influx as well as massive necrosis (Bien et al., 2011). More recently, Wilke and Bubeck Wardenburg (2010) demonstrated an interaction of a disintegrin and metalloprotease 10 (ADAM10) and α-toxin allowing binding to eukaryotic cells. The authors reported the ADAM10- α-toxin complex triggers a signalling cascade that mediates focal adhesion (Wilke and Bubeck Wardenburg, 2010).

Staphylococcus aureus β toxin, approximately 35 kDa, is Mg^{2+} dependent toxin that is cell type specific and targets sphingomyelin, phospholipids present in host tissue membrane, hence acts as a hydrolase enzyme (Huseby et al., 2007). Due to target specificity, the toxin does not lyse most cell types but renders host cell susceptible to other toxins such as α-toxin and Panton-Valentine leucocidin (PVL) (Bien et al., 2011).

The haemolysins are classed in the staphylococcal cytolytic toxin group which also includes PVL (Bocchini et al., 2006, Anderson et al., 2012b). The PVL is more toxic to leucocytes whereas haemolysins are toxic to host red blood cells (Lin and Peterson, 2010).

1.2.2 Biofilm formation

A pre-requisite for establishment of many bacterial infections, and *S. aureus*, is the ability for free floating bacteria to colonize and congregate on host surface and encase themselves in a slime layer known as biofilm. This biofilm is made of a self-secreted polymeric extracellular matrix (ECM) and consists of mainly polysaccharide and protein (Cramton et al., 1999, Smith et al., 2010, Anderson et al., 2012b, Stewart and Costerton, 2001). The composition of ECM can vary depending from strain to strain and factors such as incorporation of external particles, blood components and minerals taken from the environment (Knetsch and Koole, 2011).

A scanning electron microscopy (SEM) picture of initial attachment bacteria, to an aluminium stub, that are clumping together and secreting ECM indicated by red arrows shown in Fig 2.

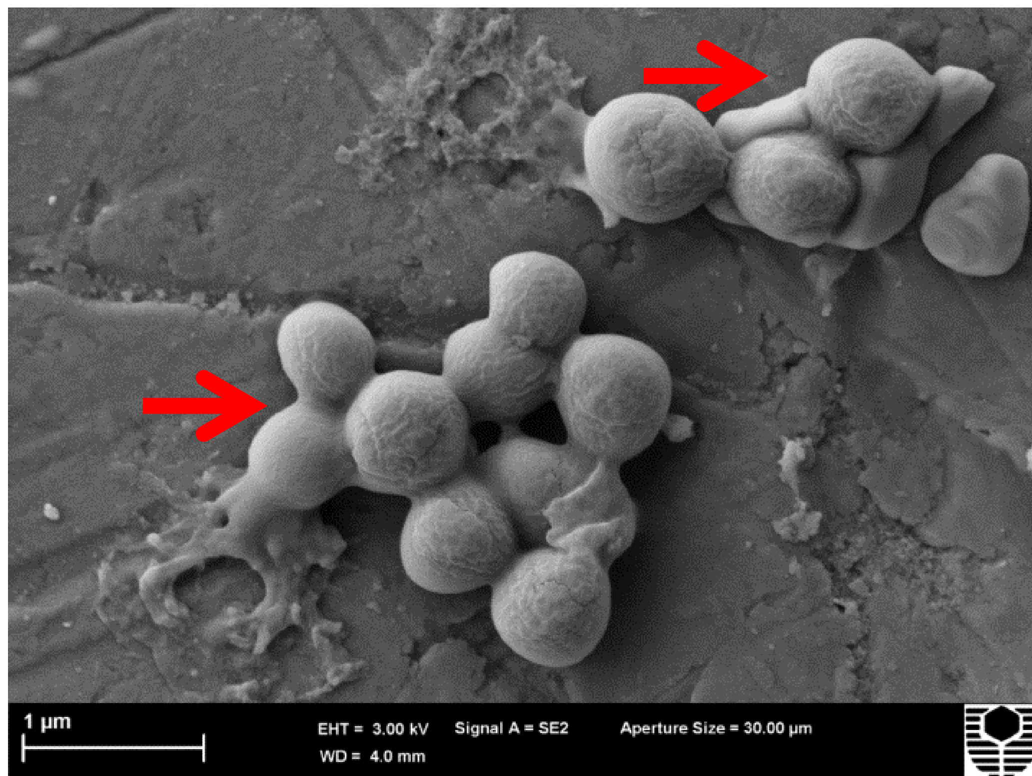


Figure 2: An SEM (x6000) of *S. aureus* cells forming a monolayer, with secretion of the protective slime layer indicated with the red arrows. Source: Image taken by Charlene Waryah, this study.

1.2.2.1 Steps of biofilm formation

Figure 3 depicts the 3 developmental stages of biofilms of *S. aureus*; attachment, maturation and dispersion (Otto, 2009). The aggregation of biofilm is significant feature as this ensures the bacteria survival and physical defence mechanism from host innate immune system defences and antibiotic therapy (Vasudevan et al., 2003, Stewart and Costerton, 2001). This restriction of penetration is a physical barrier especially against larger molecules in addition to providing a negative charge thereby repelling positively charged antibiotics and molecules such as antimicrobial peptides and complement from binding (Lewis, 2001). Biofilm also provide the bacteria with a steady yet slow growth rate, an important feature as some antibiotics are effective in killing rapidly growing bacterial cells (Lewis, 2001).

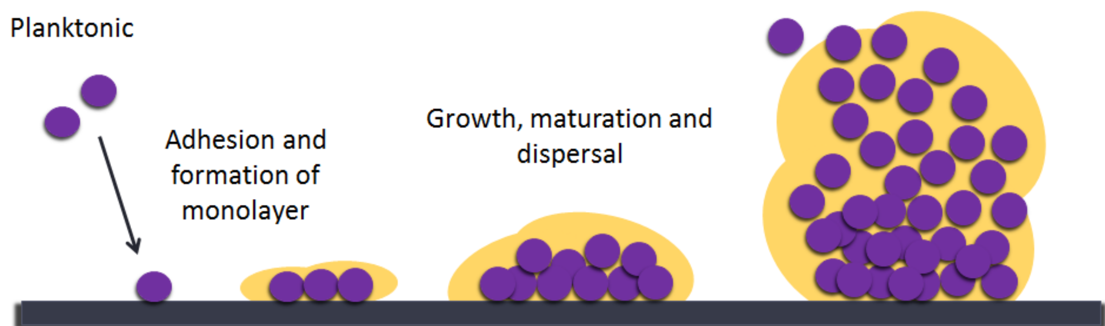


Figure 3: Development of *S. aureus* biofilm: Planktonic cells attach to host and form a monolayer. The attached monolayer secretes ECM (yellow) allowing a slime layer to develop providing an encased protection layer. The ECM continues to grow as more bacterial cells proliferate forming a mature biofilm. The final stage in biofilm development is dispersal of loose cells which become planktonic in solution and continue the cycle (Otto, 2009). Source: Image created by Charlene Waryah, this study.

The developed biofilm is not a solid layer but maintains a porous morphology allowing nutrients and oxygen to travel through holes and tunnels (Knetsch and Koole, 2011). Detachment of cells from biofilm is a crucial step for re-colonization

at a different site and can occur due to external forces such as blood flow or ECM degrading enzymes (Otto, 2008).

Biofilms are of particular interest as approximately 80% of human infections are caused by biofilm bound bacteria which are not 50 to 1000 times more resistant to antibiotics and antimicrobials as compared to their free floating form, but also tend to acquire resistance to other antibiotics (Zakrewsky et al., 2014, Babra et al., 2013a, Babra et al., 2013b). Biofilm infections caused by *S. aureus* in endocarditis results in growth on indwelling devices such as heart valves and catheters which are particularly hard to treat (Lewis, 2001, Otto, 2008).

1.2.3 Capsular Polysaccharide

Capsules are produced by several bacterial species and are composed of high molecular weight polysaccharides that attach to the cell surface (Chan et al., 2014). The polysaccharide consists of repeat units that vary from species to species and have unique structural diversity (Chan et al., 2014).

Staphylococcus aureus produces a capsular polysaccharide (CP), a virulence factor in the form of an extracellular matrix that protects the bacterium from invading host innate immune defences including different types of phagocytes (O'Riordan and Lee, 2004). Capsule production was first described in 1931 by Isabelle Gilbert and the detection methods were regarded as inadequate, with many strains of *S. aureus* being typed a non-encapsulated (von Eiff et al., 2007, Gilbert, 1931).

Based on serological specificity by immunodiffusion developed in 1982, by Karakawa and Vann (O'Riordan and Lee, 2004), 11 different capsular types were initially described for *S. aureus* (Sompolinsky et al., 1985). However, the capsular types were reduced to 4 capsular types based on gene sequencing (CP1, CP2, CP5 and CP8) and one surface polysaccharide 336 antigen (t336) (O'Riordan and Lee, 2004, Verdier et al., 2007). The of CP types of *S. aureus* are encoded by a 17.5kb region with 97 to 99% identity between serotypes (Chan et al., 2014). This region

includes 16 highly conserved genes *capABCDEFGHIJKLMNOP (*Fig 2) which include the 4 genes that specify diversity seen between the serotypes, *capHIJK* (Chan et al., 2014). The existence of CP types that were previously reported were considered to be mutants of one or more of the genes types encoding the 4 capsular types (O'Riordan and Lee, 2004, Fattom et al., 1998).

All 4 of the *S. aureus* capsular types have been purified and characterized structurally yet CP5 and CP8 account for 25 to 50% of human isolates, respectively (O'Riordan and Lee, 2004). The structure of CP5 and CP8 share same repeat units, ManNAcA, L-FucNAc, and D-FucNAc, and 12 of the 16 genes are nearly identical between the clusters (O'Riordan and Lee, 2004) as demonstrated in Fig 4.

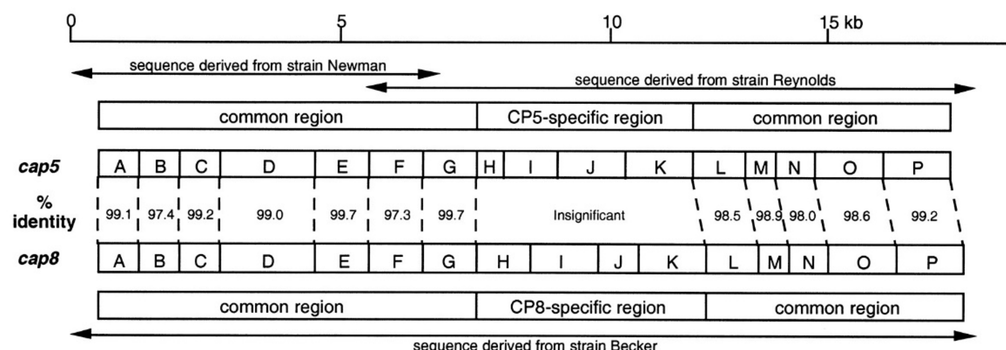


Figure 4: The cap5 and cap8 loci are allelic and share a high degree of similarity however in the *capHIJK* region; specificity is determined and distinguishes CP5 and CP8 with little homology in the regions. The cap5 gene was derived from strains Newman and Reynolds and compared with the cap8 gene derived from strain Becker. Source: O'Riordan & Lee (2004), Clin Microb Reviews Vol 17:1 pp 229.

The capsule has been found to be non-immunogenic in test trials in mice due to T-cell independence but shown to induce IgG antibodies upon conjugation with a carrier protein due to its conversion to T-cell dependence (O'Riordan and Lee, 2004, Ohlsen and Lorenz, 2010, Fattom et al., 1996, von Eiff et al., 2007).

Strains that do not test positive CP1, CP2, CP5 or CP8 were labelled as nontypeable or t336. Type336 strains do not express capsule but a variant of polyribitol phosphate *N*-acetylglucosamine, a surface polysaccharide (von Eiff et al., 2007).

1.2.4 Surface Poly-N-acetyl-(1,6)- β-D-glucosamine

Poly-N-acetyl-(1,6)-β-D-glucosamine, commonly known as PNAG, is a major contributor to the formation of biofilm. It has been identified as a high molecular weight and highly acetylated polymer of the β-1-6-linked glucosamine (Maira-Litran et al., 2005). PNAG is a key component in biofilm formation by *S. aureus* and is involved in host surface adhesion, the initial step for biofilm development (Otto, 2009, Cramton et al., 1999, Ohlsen and Lorenz, 2010). Studies have demonstrated the importance of PNAG for biofilm formation *in vitro* using various animal models (Otto, 2009).

An attractive vaccine target, PNAG is not only present in *S. aureus* but is seen in majority of coagulase negative staphylococci also as polysaccharide intercellular adhesion or PIA (Maira-Litran et al., 2005). Homologs of PNAG and PIA are also observed in a variety of other pathogens that form biofilm (Maira-Litran et al., 2005). Production of PNAG/PIA is regulated by the proteins IcaA, IcaD, IcaB and IcaC, products of the single *icaABDC* operon (Otto, 2009). The membrane anchored N-acetylglucosamine transferase IcaA and accessory IcaD genes encoding the synthesis of a partially deacetylated β 1-6 linked N-acetylglucosame polymer. The PNAG/PIA polymer is exported through the membrane IcaC protein and de-acetylated by the surface located IcaB de-acetylase protein. The deacetylation of the PNAG residues is of significant biological importance as it provides a cationic (positive) charge, essential for attachment to host surface (Otto, 2009, Vergara-Irigaray et al., 2008).

Interestingly, there is limited distribution of the *icaC* encoding gene for IcaC in gram-positive bacteria, including *S. aureus* (Atkin et al., 2014). The role this product has been identified as specific in a selected number of Staphylococcal species and is

not conserved with bacteria lacking *icaC* gene still able to produce PNAG (Atkin et al., 2014).

1.2.5 Teichoic Acid

Teichoic acids that are anchored to the cell wall are referred to as wall teichoic acids (WTA). Teichoic that are linked to cell membrane via a lipid anchor are referred to as lipotechoic acid (LTA) (Vergara-Irigaray et al., 2008, Otto, 2008). *Staphylococcus aureus* teichoic acid, a negatively charged molecule, is composed of 40 ribitol phosphate units resulting in a long chain that binds the positive charged amino groups on the PNAG covalently bind to the negative charge of teichoic acids (Fournier and Philpott, 2005). This anchoring PNAG to the cell wall of *S. aureus* hence increasing binding of bacteria to host cells (Vergara-Irigaray et al., 2008).

1.2.6 Microbial Surface Component Recognizing Adhesive Matrix Molecules

Microbial Surface Component Recognizing Adhesive Matrix Molecules, referred to as MSCRAMM are a family of surface-associated adhesion proteins of *S. aureus* (Ohlsen and Lorenz, 2010) that are expressed by *S. aureus* during exponential growth phase (Gordon and Lowy, 2008). As the name suggests, a MSCRAMM is determined by their location on the bacterial cell surface, ability to interact with a host cell receptors and thus aiding in adhesion and colonization (Harris et al., 2002). A total of 24 surface proteins, either anchored covalently to the peptidoglycan or non-covalently associated with the cell wall, with many un- identified or characterized MSCRAMM (McCarthy and Lindsay, 2010, Walsh et al., 2008, Foster, 2002). MSCRAMM that are anchored to the cell wall are acknowledged by C-terminus motif comprising of conserved LPXTG (Leu-Pro-X-Thr-Gly) sequence that recognized by an extracellular surface transpeptidase, Sortase A (Foster, 2002, Mazmanian et al., 1999). Sortase A cleaves the Thr-Gly residue which results in linkage between the carboxyl group of Thr to the amino group of peptidoglycan and provides the important C-terminus for protein anchoring (Mazmanian et al., 1999).

1.2.6.1 Protein A (SpA)

Protein A is a significant MSCRAMM anchored on the bacterial cell surface and its role has been analysed in great detail. It can promote immune evasion as it is an immunoglobulin-binding protein secreted by *S. aureus* during growth to bind to the Fc region of most IgG isotypes protecting the bacteria from opsonophagocytic killing (Kobayashi and DeLeo, 2013, Falugi et al., 2013). The *S. aureus*-bound IgG is unrecognizable by host polymorphonuclear leukocytes that renders the host immune system unable to clear infection (Foster, 2002). Protein A also is thought to B-cell receptor Fab regions triggering rapid supraclonal expansion leading to B-cell apoptosis (Collignon and Cruickshank, 2009). Figure 5 summarizes the ability of SpA to prevent opsonophagocytic killing and bacterial phagocytosis, which provides the bacteria with an immunological disguise

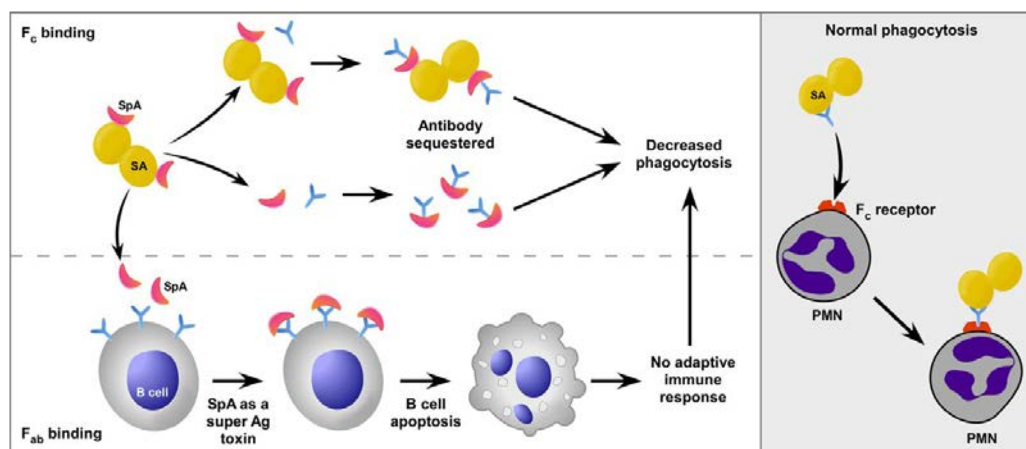


Figure 5 illustrates SpA binding to Fc domain (top) decreasing opsonophagocytic killing by inactivation of antibody response. SpA also binds to the Fab region on B cells creating a superantigen like toxin leading to B cell apoptosis hence decrease in *S. aureus* clearing (Kobayashi and DeLeo, 2013). Source: Kobayashi & DeLeo (2013) mBio Vol 4:5 pp2.

Although the protein is known primarily for binding to B cells and IgG, Spa has also been recently shown to adhere *S. aureus* to Von Willebrand factor, a blood glycoprotein important in maintaining haemostasis (Foster, 2002).

1.2.6.2 Fibronectin-binding proteins

Fibronectin binding proteins, FnBP, as the name suggests, bind to the large glycoprotein fibronectin located in soluble plasma and the insoluble extracellular matrix of eukaryotic tissue (Brouillette et al., 2003, Jonsson et al., 1991) and has been demonstrated to have a role in *in vivo* infection models (Brouillette et al., 2003). *Staphylococcus aureus* strains can have one or the two forms of FnBP, FnBPA and FnBPb, both of which have similar structure and organization (Meenan et al., 2007). The FnBPs are anchored to the hydrophobic cell wall by a LPXTG motif that aids in the adhesion to fibronectin which indirectly binds bacteria to integrins in host tissue (Meenan et al., 2007, Matthews and Potts, 2013). This is illustrated in Figure 6.

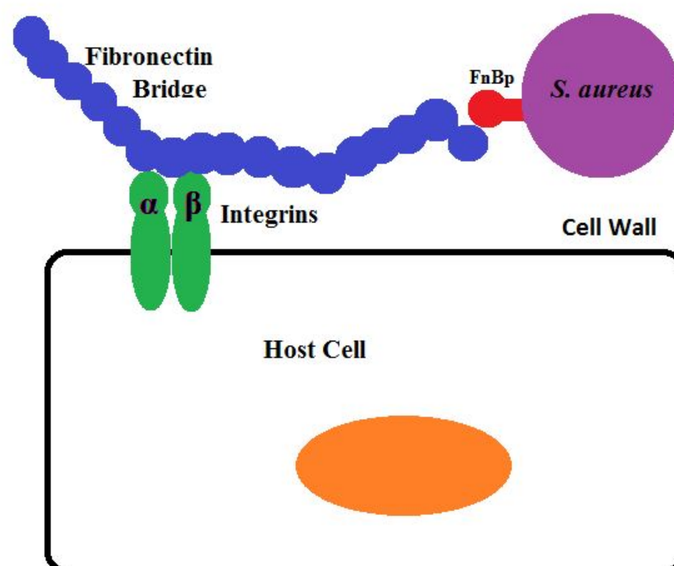


Figure 6: *S. aureus* FnBP promotes attachment to fibronectin which binds to $\alpha_5\beta_1$ integrin, creating indirect bridge for bacteria and host cell interaction (Foster, 2002). Source: Image created by Charlene Waryah, this study.

Seven isotype variants (I – VII) of FnBPa have been identified based on differences in the amino acid sequences in their ligand binding sub domains resulting in variation of antigenicity (Loughman et al., 2008). In addition to the differences in antigenicity, the domains exhibit limited immunocross-reactivity and are between 66-76% identical in amino acid sequences (Loughman et al., 2008). Seven different allelic variations of FnBPb have also been identified and are 61-85% identical based on their amino acid sequence (Burke et al., 2010). The isoforms of FnBPb display differences in antigenicity and are able to retain ligand binding capability (Burke et al., 2010).

1.2.6.3 Clumping Factors A and B (ClfA and ClfB)

Staphylococcus aureus can also express proteins that can specifically bind to fibrinogen, a plasma glycoprotein involved in haemostasis (formation of blood clots) (Walsh et al., 2008). These include clumping factors A (ClfA) and B (ClfB) and have also been demonstrated to promote bacterial clumping in the presence of fibrinogen (Walsh et al., 2008, McDevitt et al., 1997).

Fibrinogen is composed of three polypeptide chains – α, β and γ, ClfA binds to the γ chain whereas ClfB binds to the β chain (Ni Eidhin et al., 1998, McDevitt et al., 1997). The affinity of ClfA/ClfB to fibrinogen is high, resulting in clumping even at low concentrations of the plasma protein (Ni Eidhin et al., 1998). The presence of ClfA expression in infection has been identified as an important MSCRAMM for the development of septic arthritis (Foster, 2002).

1.2.6.4 Collagen Binding Protein (Cna)

Collagen is an important and abundant protein that provides structure to connective tissue in animals (Svensson et al., 2001). The collagen binding MSCRAMM or Cna is composed of two regions, one of which is a non-repetitive A region that contains a collagen binding site (Deivanayagam et al., 2000). It binds to collagen with

moderate affinity however has been show to play an important role in *S. aureus* pathogenesis in septic arthritis and infective endocarditis (Deivanayagam et al., 2000, Hienz et al., 1996).

1.2.6.5 Manganese Transport Protein (Mnt)

One group of proteins that are gaining attention in their roles as MSCRAMM are a complex of proteins known as the manganese transport proteins (Mnt), ATP-binding cassette transporter complex (Anderson et al., 2012a). The complex consists of three proteins: ATP-binding protein MntA, an integral membrane transporter MntB and manganese binding lipoprotein MntC (Anderson et al., 2012a). During bacterial infection, host cells can deprive bacteria by restriction of important metal ions (Horsburgh et al., 2002). The uptake of manganese for bacterial survival is essential as it is involved in catabolism and metabolism of *S. aureus* (Horsburgh et al., 2002). The MntC has drawn attention as it is highly conserved between *S. aureus* and its orthologous protein SitC in *S. epidermidis,* in addition to this protein's early expression during infection (Anderson et al., 2012a).

1.2.6.6 Other MSCRAMM

Two iron-regulated surface determinants, IsdA and IsdB can also be expressed by *S. aureus* during infection ((Kim et al., 2010b, Mazmanian et al., 2002). The proteins are essential for *S. aureus* survival in blood and increases virulence in abscess formation (Kim et al., 2010b). The acquisition of iron by bacteria from the host environment is a key step in establishing infection with blood haemoglobin as a common source (Pishchany et al., 2009).

The Sdr proteins, SdrC, SdrD and SdrE, are members of the *S. aureus* MSCRAMM family and contain repeating serine-aspartate dipeptide of an R region (McCrea et al., 2000, Sabat et al., 2006). The proteins are similar in structure to ClfA and ClfB, and have been shown to have high affinity for Ca^{2+} (Sabat et al., 2006) There are

predictions that the SdrE promotes platelet aggregation and Bbp, a variant of SdrD, that can bind to the bone sialoprotein (Foster, 2002).

1.3 Immune response to infection with *Staphylococcus aureus*

In order to develop strategies for the prevention of staphylococcal infections, it is important to understand the potential roles that innate and adaptive immune mechanisms, particularly against biofilm-embedded antigens, can play.

1.3.1 Innate Immunity

As *S. aureus* is part of the normal microflora of the skin, a compromise in the integrity of the tissues cause by abrasion or that associated with invasive medical devices (Krishna and Miller, 2012), may result in the onset of infection at the target site leading to the triggering of the onset of host inflammatory response with an escalated production and release of proinflammatory and immunoimodulating cytokines, chemokines and antimicrobial peptides (Krishna and Miller, 2012). The production of cytokines is essential for initiation of innate immune response including attraction of neutrolphils, NK cells and dendritic cells to the sight of infection. A list of the types of cytokines produced is shown in Table 2.

Table 2: A summary on the types of cytokines and their functions

Family	Cytokines	Effect
Interleukin	IL-1α, IL-1β	Activates TNF-α, important mediators of inflammation
	IL-6	Pro and anti-inflammatory cytokine; enhanced activity of TNF and IL-1α
	IL-10	Anti-inflammatory cytokine, suppresses activity of Th1 cells, NK cells and macrophages to prevent tissue damage
	IL-12	Th1-skewing promoted
Cytotoxic cytokines	TNF-α, TNF-β	Enhance proliferation and differentiation of lymphocytes, stimulate other cytokines hence mediators of septic shock
Chemokine	IL-8	Induces chemotaxis for neutrophils and other granulocytes; stimulates angiogenesis
Interferon	IFNα, IFN-β, IFN-γ	Antiviral activity, regulators of the innate immunity
Colony stimulating factors	CSF1, CSF2, CSF3	Stimulate the formation of macrophage colonies

Ref: Adapted from (Coico and Sunshine, 2009, Tisoncik et al., 2012, Fournier and Philpott, 2005, Couper et al., 2008)

Initiation of pro-inflammatory factors begins with the recognition of pathogens associated molecular patterns (PAMPs) by the appropriate pathogen recognition receptors (PRRs) (Fournier and Philpott, 2005).

Toll-like receptors (TLR) are a major subgroup of PRRs that initiate interaction between the host immune cells and PAMPs leading to initiation of the adaptive immune responses against the microbial pathogens. Different types of TLRs are expressed surface of the antigen presenting cells with TLR4. Following bacterial infection, TLR4 mediate interaction with gram-negative bacteria and TLR2 mediate interaction with gram positive-bacteria (Wang et al., 2012). The bacterial cell wall components recognized include peptidoglycan, lipoproteins and lipoteichoic acid, found in gram-positive bacteria (Fournier and Philpott, 2005, Thurlow et al., 2011). Macrophages and dendritic cells also phagocytize the pathogens initiating adaptive immune responses to the recognized PAMPs (Fournier, 2012, Hanke and Kielian, 2012). TLR2 has also been largely accepted as a key recognition receptor associated with the attraction of neutrophils as a result of the interaction with *S. aureus* lipoprotein and peptidoglycan in the phagosome (Fournier, 2012).

Biofilms of *S. aureus*, contain polysaccharides, proteins and eDNA (Thurlow et al., 2011). Bacterial DNA and biofilm eDNA can engage TLR-9-dependent activation (Thurlow et al., 2011) resulting in attraction of the phagocytes including macrophages, neutrophils and dendritic cells amplifying the process of phagocytosis, and initiation of the adaptive immune response.

1.3.2 Acquired Immunity

It is beyond the scope of this literature review to describe the details of the immunological events underpinning the initiation of adaptive immunity which has been described in multiple immunology/immunobiology textbooks and reviews (Coico and Sunshine, 2009, Murphy et al., 2012). A brief description is however described below.

The cytokines produced post-interaction of the innate immune associated phagocytic cells such as dendritic cells, macrophages particularly IL-12 and IFN-γ, result in the initiation of the development of adaptive immunity (Janeway and Medzhitov, 2002).

Naïve T-cells, which continuously circulate and migrate within the host awaiting activation by allowing for interaction with antigen-specific epitopes presented to them by dendritic cells in the secondary lymphoid organs, develop as either helper T cells (T_H1, T_H2) that interact with MHC Class II or cytotoxic T cells (T_C) that interact with MHC Class I. Each T cell subset produces different types of cytokines (Table 3).

T_H1 cells primary produce IFN-γ, IL-2 and TNF-β whereas T_H2 cells produce IL-3, IL-4, IL-5, IL-10 and IL-13. These 2 subsets also differ from each other in their responses of infection with intracellular verses extracellular pathogens. T_H1 cells respond to the intracellular pathogens whereas T_H2 cells respond to the extracellular pathogens. Mysteriously, each T_H subset performs the task of stimulating the production of each subset while inhibiting the development of the other T-cell subset through the cytokine IL-10 produced by T_H2 cells and INF-γ produced by T_H1 cells. However, the mechanisms of differentiation of T-cell precursors into one of the other T cell subsets *in vivo*, is still not known. The current hypothesis is that the cytokine environment of the naïve T cells differentiation promotes induction of one or the other subset. In the presence of IL-12, the naïve T cells differentiate into T_H1 whereas in the IL-4 environment, they differentiate into T_H2 subset. The major cells types and the effector molecules produced at shown in Table 6. The balance of cytokines and bacterial antigen presented to T-cells can dictate the directions of the immune response toward cellular and/or humoral immunity.

Table 3: Production of cytokines by different lymphocyte subsets*

Subsets of $CD4^+$ T cell	Cytokines produced	Major functions
T_H1	IL-2	Major growth factor, induces proliferation and differentiation of T cells and NK cells
	IFN-γ	Primary role is to control neutrophil recruitment and trafficking, differentiates T cells into T_H1
	TNF-β	Phagocytosis
T_H2	IL-3	Stimulates cell proliferation and differentiation, progenitor
	IL-4	Stimulates cell proliferation, isotope (IgE) switching and up regulates production of MHC class II, inhibits $T_H1$production
	IL-5	Regulations the activation, proliferation and differentiation of eosinophils
	IL-10	Inhibits IFN-γ synthesis, down regulates expression of cytokines
	IL-13	Inhibits inflammatory cytokines
T_C	IFN-γ	Primary role is to control neutrophil recruitment and trafficking, increases expression of MHC class I proteins in bacterial cells allowing for recognition of cytotoxic attack, activates macrophages
	TNF-β	Coordinates with IFN-γ for activation of macrophage

* Khan, 2008, McLoughlin et al., 2008, Murphy et al., 2012

The responses of B cells to antigens depend upon the nature of the antigens. Polysaccharides are T-cell independent (T-I) not requiring the help of T-cells. These antigens are classified as type II antigens because the immune response stimulated are specific but not polyclonal because of the engagement of B-cell receptor.

T-I type II antigens induce strong primary antibody responses in mice which has been claimed to confer long-term humoral immunity (Garcia de Vinuesa et al., 1999), generate memory B-cells and stimulate extrafollicular foci for plasma cell production (Obukhanych and Nussenzweig, 2006).

Protein antigens are T-dependent (T-D) antigens that are processed for presentation of the epitopes displayed in the MHC Class II molecules for interaction and recognitions by the helper T_H2 and/or T_H1 cells. T-D antigens stimulate germinal centres which can be identified by expression of IgG, IgE or IgA isotypes or somatic hypermutations in the Ig loci (Kocks and Rajewsky, 1989, McHeyzer-Williams and McHeyzer-Williams, 2005).

1.4 Current approaches to *S. aureus* therapeutics

1.4.1 Active vs Passive immunisation

Passive immunization occurs when antibodies produced against an antigen of interest are administered to the subjects (Lee, 2003). The benefit is for immunocompromised patients, particularly in ICUs or neonates who cannot generate an immune response for the clearance of a bacterial infection (Rauch et al., 2014). Target groups also include patients undergoing surgery such as emergency or implanted devices and trauma victims who may not be able to produce adequate immune responses quick enough by active immunisation and are limited to time constraints and need a quick response (Schaffer and Lee, 2008, Berg and Bakker-Woudenberg, 2013). Passive immunotherapy will provide an immediate response, however injected antibody will be quickly broken down and thus will provide short term protection (Berg and Bakker-Woudenberg, 2013).

Active immunization refers to self-production of immune responses including the production of antibodies and/or CMI depending upon the nature of the antigen and adjuvant used, following exposure to antigens. This immune response maybe naturally acquired or artificially acquired (Berg and Bakker-Woudenberg, 2013). Unlike passive immunization, acquired immunity lasts a longer time. The use of an adjuvant is frequently preferred to induce sufficient T cell-dependant immune response (Berg and Bakker-Woudenberg, 2013).

The most commonly used adjuvants are alum-based which preferentially induce T_H2 polarized immune responses with little contribution to the induction of T_H1 mediated immunity upon the route of immunization (Feinen et al., 2014). More recently, alum-based adjuvants formulation combined with the relevant TLR agonists have been reported to yield T_H1 polarized immune response and improved protective potential against the disease syndromes (Allen and Mills, 2014).

The target groups for active immunization with an effective *S. aureus* vaccine may include patients with pending elective surgeries, high risk transfer groups such as healthcare providers, school children, athletes, prisoners and military personnel, as

well as intravenous drug uses or individuals with long term ailments such as HIV and diabetes (Schaffer and Lee, 2008).

1.4.2 Current status of Vaccine Development against *S. aureus*

There is currently no successful *S. aureus* vaccine available. However several research groups around the world are investigating targets for active and passive immunisation (Daum and Spellberg, 2012, Shinefield and Black, 2006, Bagnoli et al., 2012).

1.4.2.1 Whole cell vaccine

This vaccine consists of heat or formalin-inactivated whole cells mixed with an alum-based adjuvant or Freund's incomplete adjuvants, the latter being used only in animals for the prevention of bovine mastitis. The key advantage to a whole cell vaccine is a production of antibodies against a broad spectrum of antigens present in *S. aureus*. Vaccine Research International has developed a whole cell vaccine, SA75 that is currently in Phase I trials (http://www.vri.org.uk/PhaseITrial.pdf). The vaccine contains whole *S. aureus* killed in chloroform and has been shown to stimulate an immune response of IgG in 75% of patients (24 of 32 subjects), while being safe to administer to humans (Ohlsen and Lorenz, 2010). A phase I clinical trial showed 64% of vaccinated individuals had antibodies against Cna, low response towards clumping factor (3%) and no antibodies towards FnBP and Eap (Ohlsen and Lorenz, 2010). However in late 2006, the project stalled due insufficient funds and vaccine trials are no longer progressing (Berg and Bakker-Woudenberg, 2013).

1.4.2.2 Capsular polysaccharide-based vaccines

Capsule polysaccharides have been used successfully for the development of vaccines against infections with *Haemophilus influenzae* type b, *Neisseria*

meningitidis and *Streptococcus pneumoniae* and are a popular target for vaccine development due to their importance in virulence (Huda et al., 2011) *Staphylococcus aureus* capsular polysaccharide has proven to be ineffective as demonstrated by their poor immunogenicity in a mouse model (Fattom et al., 2004).

Nabi Biopharmaceuticals conjugated CPs types 5 and 8 with *Pseudomonas aeruginosa* exotoxoid A (StaphVax) vaccine which failed in Phase III trials in haemodialysis patients (Proctor, 2012). However, there was some success post vaccination with the capsular conjugate vaccine in reducing *S. aureus* bacteraemia using CP-targeted antibodies up to 10-14 day post immunisation (Schaffer and Lee, 2008). By the end of the trial, at the end point of week 54, there was a 26% reduction of *S. aureus* bacteraemia (Walsh et al., 2008). Further developments with StaphVax stopped and lead Nabi Biopharmaceutical to test it as a potential passive immunotherapy vaccine called AltaStaph. AltaStaph consists of polyclonal antibodies against CP5 and CP8 derived from healthy individuals who received the StaphVax preparation (Fattom et al., 2004). Current reports from the AltaSaph phase II clinical trials have failed in preventing bacteraemia in neonates and deaths in children and adults with bacteraemia (Otto, 2010a). Nabi Pharmaceuticals have halted both as the trials have proven unsuccessful in protection to patients with *S. aureus* infections (Otto, 2010a).

Nabi Pharmaceuticals are currently evaulating TriStaph™ and PentaStaph™. TriStaph™ includes type 336 in addition to CP5 and CP8 (Huda et al., 2011) *P. aeruginosa* exotoxoid A. PentaStaph™, a 5 component vaccine conjugated to *P. aeruginosa* exotoxoid A, targets CP types 5 and 8, type 336, PVL and alpha toxin as potential vaccine candidates. This vaccine is currently in Phase II trials (Ohlsen and Lorenz, 2010, Proctor, 2012). A similar 4 component vaccine, SA4Ag, is being developed by Pfizer which includes CP5 and CP8 individually conjugated to a recombinant ClfA and MntC (Peter Richmond, Head of Vaccine Clinical Trials Group, Telethon Kids Institute, Western Australia, confidential personal communication).

Some reasons for the lack of success in the development of CP based conjugate *S. aureus* vaccines that have been put forward include production/induction of non-efficacious antibodies unlike the protective antibodies produced by conjugate vaccines against infections with *H. influenzae* type b, *N. meningitidis* and *S. pneumoniae* (Daum and Spellberg, 2012, Huda et al., 2011). Another potential reason for the failure of CP-based vaccines may be due to the existence of significant numbers of non-encapsulated *S. aureus* strains as exemplified by the USA300 strain, a currently circulating highly virulent CA-MRSA strain (Daum and Spellberg, 2012).

1.4.2.3 PNAG-based vaccine

PNAG is currently in preclinical trials for potential vaccine development (Ohlsen and Lorenz, 2010, Harro et al., 2010). The native form of PNAG, which is acetylated, was trialled however the antibodies generated in a *S. aureus* bacteraemia mouse model were unsuccessful in clearing infection (Schaffer and Lee, 2008). Naturally occurring PNAG is enzymatically modified to lose its cationic character resulting in a partial deaceytlated form, shown to have a crucial role in structural integrity of the polymer and biological function (Otto, 2010a, Vuong et al., 2004). Maira-Litran et al, (2005) raised antibodies against dPNAG conjugated to diphtheria toxoid and trialled as a potential passive immunotherapy using mouse as an animal model to determine its potential to prevent of sepsis. Mice injected with dPNAG specific antibodies showed reduction of 54-91% of *S. aureus* in blood culture than controls (Maira-Litran et al., 2005).

1.4.2.4 Lipoteichoic acid based vaccine

Biosynexus Inc developed Pagibaximab, a passive immunotherapay vaccine composed of human chimeric monoclonal antibodies against lipoteichoic acid developed by recombinant DNA technology (Weisman et al., 2011). As lipoteichoic acid is present in all gram-positive bacteria, the vaccine aimed at preventing

bloodstream infections against *S. aureus* and coagulase negative staphylococcal species in low birth weight neonates (Schaffer and Lee, 2008).

Pagibaximab antibodies have been bind to *S. aureus* lipoteichoic acid and subsequent cytokines produced following infection (Weisman et al., 2011). Published reports have indicated success in neonates with regards to its safety and potential of protective antibodies. Howeve, no data obtained from the PhaseII/III trials have been published as yet (Huda et al., 2011, Jansen et al., 2013).

1.4.2.5 MSCRAMM

Vaccines targeting other MSCRAMM are also under investigations for their vaccine potential against staphylococcal infections in different laboratories (Jansen et al., 2013, Maira-Litran et al., 2005, Bagnoli et al., 2012, Kim et al., 2010b, Pozzi et al., 2012).

Infection with a *S. aureus* mutant strain demonstrated that loss of IsdB *in vivo* significantly decreases virulence and hence considered as an attractive vaccine target as it is conserved among clinical isolates (Kuklin et al., 2006). IsdB was first identified during a study where patient sera were screened for antibodies against *S. aureus* (Kuklin et al., 2006). Due to recent interest in the proteins involved in iron regulation, Syntiron/Sanofi Pasteur have patented a multivalent vaccine technology (SRP®) targeting all iron-regulating proteins (Sheldon and Heinrichs, 2012).

Merck developed Vaccine V710, containing the IsdB recombinant protein, was trialled in adults with *S. aureus* infection following elective cardiothoracic surgery (Schaffer and Lee, 2008). This vaccine initially claimed to successfully induce opsonophagocytic antibodies prior to elective cardiothoracic surgery however was unable to reduce incidence of postoperative *S. aureus* bacteraemia in the patients (Fowler et al., 2013) and resulted in higher mortalities then in the placebo group (McNeely et al., 2014). This trial has now been halted.

Inhibitex, Inc targeted the MSCRAMM proteins using polyclonal antibodies against ClfA in *S. aureus* and SdrG in *S. epidermidis* and developed the vaccine INH-A21 Veronate® (DeJonge et al., 2007). From the blood donor population, 2% of donors exhibited high levels of antibody against the antigens and were selected for production of INH-A21. Similar to their phase II clinical trials, the target group was low birth weight infants and the delivery of passive immunotherapy to prevent late onset of sepsis (DeJonge et al., 2007). Unfortunately, in a total of 50 of 989 placebo infants (5%) and 60 of 994 vaccine administered infants (6%) developed late onset of sepsis indicated no statistically significant differences between the vaccinated verses the placebo groups (DeJonge et al., 2007).

Inhibitex, Inc has also developed a monoclonal antibody, called tefibazumab (Aurexis), with high affinity against ClfA (Ohlsen and Lorenz, 2010, Proctor, 2012). Endocarditis rabbit models used to test the passive protective potential of two doses of tefibazumab in combination with vancomycin when successful reduction in *S. aureus* levels in blood and organs was found.

Based on molecular modelling, recombinant N terminus of the *Candida* adhesion protein Als3p or rAls3p-N reported three dimensional structural similarities to *S. aureus* ClfA (Spellberg et al., 2008). The rAls3p-N has been was found to protect mice against lethal candidiasis but only when used as a vaccine conjugated to complete Freund's adjuvant. This vaccine induced a high T cell mediated immune response and protected animals against *S. aureus* lethal challenge. Baquir et al., (2010) further tested the immunogenicity of this vaccine and confirmed the rAls3p-N was able to stimulate IFN-γ and IL-17, requirements for protection in mice. While the authors are planning for phase I clinical trials (Baquir et al., 2010), it is important to note that the use of Freund's complete adjuvant in humans or even in animals is not permitted by the FDA. Therefore, alternative more suitable adjuvants will need to be tested.

Two combination vaccines, ClfA, FnBPB, and SdrD as combo 1 and ClfA, FnBPB, SdrD and a non-toxigenic SpA_{KKAA} (a mutant of Spa) as combo 2 were investigated by Kim et al. (2011). Combinations were mixed with either complete and/ or

incomplete Freund's adjuvant. Both vaccines resulted in a significant reduction in bacterial load following challenge with *S. aureus* strain Newman (Kim et al., 2011). Mortality rates were delayed that was statistically significant over the mock challenge mice which succumbed to infection on day 4 post challenge (Kim et al., 2011).

Kim et al., (2010) engineered a variant of Spa with a mutation in its binding domains to Fcγ or Fab V_H3 to generate SpA_{KKAA}, a mutant unable to bind to the immunoglobulins and unable to generate a B-cell apoptosis (Plotkin et al., 2013). When Balb/c mice in the animal trials were injected with the purified mutant SpA-D_{KKAA} emulsified in aluminium hydroxide adjuvant, the antibody tires found to be 10-fold higher than the non-variant (Kim et al., 2010a). When challenged with *S. aureus* strain Newman, the antibodies produced were able provide protection by decreasing infection and increasing opsonophagocytic clearance (Kim et al., 2010a). Overall, mice vaccinated with Spa_{KKAA} displayed fewer staphylococci in organ tissue after challenge and reduced mortality rates leading to interesting results for the potential use of Spa as a vaccine target (Kim et al., 2010a).

1.4.2.7 Exotoxins

A recent study demonstrated successful active immunization using a mutant form of Hla (Hla_{H35L}) produced by a single amino acid substitution of histidine 35 with leucine. This substitution renders toxin unable to form cytolytic pores in host cells (Bubeck Wardenburg and Schneewind, 2008). The Hla_{H35L} vaccine protection in a mouse *S. aureus* pneumonia and decreased bacterial CFU load in lung tissue of mice (Bubeck Wardenburg and Schneewind, 2008). The same group tested the anti-Hla_{H35L} antibodies in a passive immunisation mouse model and reported decrease of bacterial CFUs observed in lungs tissue ($P=0.02$). Interestingly, passive immunization with anti-PVL antibodies did not confer protection against in mice against *S. aureus* infection. In contrast, Brown et al., (2008) reported that their PVL vaccine offered protection in mice against infections with CA-MRSA. The difference between the PVL mutant strains used in this experiment was, unlike an

amino acid substitution created by Bubeck Wardenburg and Schneewind, this research group genetically replaced the PVL genes with the tetracycline resistance gene *tet*M (Labandeira-Rey et al., 2007). Similarities by both groups include the use Balb/c mouse pneumonia models with similar bacterial challenge via subcutaneous routes of injection.

Vaccines aimed at neutralizing the toxicity of SE toxins have also been evaluated using mouse as a model system. LeClaire et al., (2002) genetically altered SEB with a 3 mutations at site L45R/Y89A/Y94A producing inactivated toxin, SEBv. This mutation renders the toxin unable to interact with MHC Class II receptors (Larkin et al., 2010). The altered toxin was mixed with and/or without Ribi adjuvant and challenged in a mouse model to observe inflammatory cytokine responses. Unfortunately, while the vaccine was able to elicit a high amount of neutralizing antibodies, the antibodies were unable to suppress the T cell responses produced *in vivo*. The same group purified the anti-SEB antibodies using affinity chromatography followed by evaluation in a rhesus monkey model. Antibodies were delivered intraperitoneal 20mins prior to and 4hr after aerosolized SEB challenge. The results were deemed successful as all rhesus monkeys receiving the antibody survived while the control group monkeys succumbed to the challenge with a wild-type SEB producing strain (LeClaire et al., 2002).

Integrated BioTherapeutics are trialling recombinant SEB containing the same point mutations, L45R/Y89A/Y94A, as used by Le Claire's group (Larkin et al., 2010). Pre-clinical trials with a preparation of the polyclonal antibodies raised against the mutated antigen were found to neutralize the toxin *in vitro*. This vaccine, called STEBVax, is currently awaiting recruitment of healthy adults for phase I clinical trials for the treatment of TSS (http://www.clinicaltrials.gov/ct2/show/NCT00974935). Interestingly antibodies raised against mutant SEB have been shown to cross react with SEC, which contains 68% sequence similarity to SEB (Larkin et al., 2010). Given the low distribution among *S. aureus*, the vaccine is being development for protection should the superantigen be used for biological warfare purposes (Otto, 2010a).

1.4.3 Pre-requisites for a successful *S. aureus* vaccine

Staphylococcus aureus is a challenging target for vaccine development because of the production of multiple arrays of virulence factors, all of which vary in expression depending on strain, the environment *in vivo* and type of infection. An ideal vaccine will have to target all of the following obstacles as described by Scully et al., (2014) and Ohlsen and Lorenz, (2010).

1. Ability to prevent bacteria essential nutrients required for proliferation *in vivo* and hence survival.

2. Ability to prevent adherence of *S. aureus* by blocking interaction of potential adhesins, including structural and secreted proteins and polysaccharides to host cells.

3. Developing strategies for overcoming bacterial immune evasion and anti-phagocytosis strategies.

4. Ability to neutralize the action of the exotoxins and enzymes capable of killing the host cells including immune cells by lysis apoptosis or necrosis.

The multivalent approach to vaccine development has understandable been the preferred approach to ensure coverage of more target antigens than single antigen targets (Scully et al., 2014). Data presented in this thesis indirect supports the multivalent vaccine approach whether used for active immunisation or passive immunotherapy.

1.4.4 Anti-biofilm treatment strategies

Inhibition of biofilm formation and attachment strategies has been investigated (Ammons, 2010) and coating of medical implant devices with antibiotics has been studied (Knetsch and Koole, 2011) with valuable results due to the potential development of antibiotic resistance (Babra et al., 2013a, Babra et al., 2013b). The

aims of anti-biofilm approach is to prevent the initiation of formation of biofilm as well as to develop strategies for disruption of already formed biofilms a view to enhancing the killing efficacy of antibiotics.

Minocycline and rifampin coated catheters were tested to determine incidence of central line associated *S. aureus* bacteraemia in hospital settings and showed significant decrease (Ramos et al., 2011). Antibiotic coating of medical devices in one study involved adsorption of the antibiotic amoxicillin and rifampin onto polyurethanes which was reported to better the performance of antibacterial activity in terms of longevity (Piozzi et al., 2004).

However, despite the improvement of antibacterial activity, seceral issues arise with this treatment strategy. The first and foremost issue lies in the ability of antibiotic to successfully maintain antimicrobial activity for long-term implants (Knetsch and Koole, 2011). Short-term indwelling devices such as catheters could benefit as compared to permanent devices as minimum inhibitory concentration maintenance is more likely. The second major issue with this strategy is the potential of the medical device associated *S. aureus* to develop resistance to antibiotics to which it was previously susceptible in the planktonic state (Babra et al., 2013b).

1.4.5 Electrochemical and ultrasound treatment

Electrochemical and ultrasound approaches towards biofilm degradation have been investigated in an effort to enhance antibiotic transport through the ECM (Smith, 2005). Introduction of low power electrical currents to bacteria can affect electrophorectic mobility of membrane proteins and disorient cell behaviour and structural integrity (Del Pozo et al., 2008). Using this theory, the current can increase bacterial sensitivity with potential bactericidal effects in the presence of antibiotics. Enhanced penetration of erythromycin, daptomycin and moxifloxacin on MRSA biofilms over 24h was demonstrated *in vitro*. However, this strategy *has* yet to be explored *in vivo* (Del Pozo et al., 2008). Low levels of ultrasound frequency have been shown to increase permeability through biofilm channels. Using this principle,

low frequency ultrasound enhanced bacterial killing by gentamycin on *E. coli* biofilms and vancomycin on *S. epidermidis* biofilms (Smith, 2005). More recently, ultrasound has been reported to enhance the bactericidal of the antimicrobial peptide, human β-defensin 3, against *S. aureus* biofilm (Zhu et al., 2013).

1.4.6 Silver anti-biofilm treatment

Silver, as an antimicrobial agent, is an ancient form of treatment going back to 4000BC with the transport of water in silver vessels and silver salts for water preservations (Knetsch and Koole, 2011) and the effects of silver ions and compounds have been well acknowledged as toxic towards several microorganisms (Kim et al., 2007). More recently, silver ions and silver nanoparticles have been used successfully in the treatment of burns and chronic wounds associated with or caused by *S. aureus* (Chung and Toh, 2014). Silver ions have an effect on DNA replication, rendering bacteria unable to replicate in addition to inhibiting oxidation of glucose and products involved in the respiratory cycles (Secinti et al., 2011). Secinti *et al* (2011) tested *S. aureus* infected rabbit model over a 28 day experiment with and without nanoparticle silver ion coated implanted screws. The team discovered that all silver coated screws were uninfected whereas 90% of the uncoated titanium control screws had developed biofilm (Secinti et al., 2011). Though initial reports are promising, demonstration of the long-term effect with implanted silver coated devices is yet to be investigated.

1.4.7 Antimicrobial peptides

Antimicrobial peptides, or AMPs, are oligopeptides that were first discovered in 1939 when the soil bacteria extracts were found to exhibit antimicrobial activity against pneumococcal infections in mice (Bahar and Ren, 2013). Specifically peptides with antimicrobial activity have been isolated from various sources such as human, animal, insects, bacteria and plant cells with over 5,000 AMPs synthesized since their discovery (Bahar and Ren, 2013).

In humans, primarily keratinocytes, AMPs are part of innate immunity with activity against bacteria, fungi and viruses. The largest group of AMPs produced by human keratinocytes and sebocytes are defensins which have antimicrobial properties against gram-positive and negative-bacteria. AMPs are categorized into 1 of 4 secondary structural groups viz., β-sheet, α-helix, extended, and loop (Peters et al., 2010). The main target for antibacterials AMPs is the lipopolysaccharide found abundant in the bacterial cell membrane of gram negative bacterial pathogens. Among AMPs, antibacterial AMPs are the largest researched and investigated group (Bahar and Ren, 2013). Nisin, an antibacterial AMP, has been shown to be able to kill MRSA with higher MICs than antibiotics, chloramphenicol and ramoplanin, used in the study and similar activity to bacitracin (Brumfitt et al., 2002).

Unfortunately, resistance towards antimicrobial peptides has been demonstrated widely by staphylococci species. Both *S. aureus* and *S. epidermidis* produce secreted proteases such as areolysin which actively degrade select AMPs as well as surface charge changing proteins including *Dlt* locus which causes D-alanylation of teichoic acids and IcaB *N*-acetylglucosamine deacetylase which induces positive charge on the pathogen (Otto, 2010b, Li et al., 2007). Most AMPs display a cationic character and an alteration on surface charge discourages binding hence inhibiting antimicrobial activity (Saar-Dover et al., 2012). Bacteria have remarkably adapted to counteract the presence of AMPs by cationic AMP sensors. In addition to the *Dlt* locus, these include the *vraF/vraG* genes which encode for the ABC transporter system proteins and the *mprF* gene which encodes an enzyme that prevents binding of cationic AMP to the bacterial cytoplasmic membrane (Li et al., 2007).

Though much research *in vitro* has been performed and is still ongoing, investigations *in vivo* have yet to be carried out for evaluation of the *in vitro* observations.

1.4.8 Other

Ionic liquids (IL) and deep eutectic solvents (DESs) have been studied and are acknowledged as having antifungal and antimicrobial properties (Zakrewsky et al., 2014). More recently, Zakrewkey et al., (2014) demonstrated the use of IL and DESs in the successful treatment of skin infections *Pseudomonas aeruginosa* and *Salmonella enterica* biofilms in combination with a topical drug delivery and/or antibiotic. Treatment was delivered against infected wounds with the use of a synthesised IL and ceftazidime, a broad spectrum antibiotic. The group confirmed >98% reduction of biofilm with the dual formulation confirming the ability of IL to breakdown biofilm resulting in enhancement of antibiotic's ability to reduce bacterial numbers (Zakrewsky et al., 2014). Ionic liquids work by disrupting and lysing bacterial cells following interaction with the negatively charged bacteria cell membrane (Venkata Nancharaiah et al., 2012). Venkata Nancharaiah et al., (2012) found that IL was able to increase *S. aureus* membrane permeability *in vitro* and successfully prevent biofilm development using two biofilm assays. The activity of IL in their study was also found to be higher against gram-positive bacteria, *S. aureus*, with a higher rate of permeability and biofilm prevention as compared to gram negative bacteria, *P. aeruginosa*.

1.5 Objectives of this Research Project

The goals of this research project were:

- To identify and characterize novel surface-associated protein potential biofilm forming antigens and examine their significance as virulence and potential vaccine candidates in the prevention of systemic infection
- To explore strategies for dispersal of biofilm with a view to enhancing the efficacy of treatment with antibiotics
- To develop an *in vitro* method for identification of predominant biofilm forming antigens of *S. aureus*
- To validate the biofilm forming potential of the antigens using *in vitro* methods developed in this project
- To identify novel biofilm-asociated antigen(s) and evaluate the immunogenicity and protective potential of the major identified antigen(s)

In order to accomplish the goals stated above, the following aims need to be accomplished;

[1]. Collection, phenotypic and genotypic characterization of Australian *S. aureus* of human origin.

[2]. Identification of the major exotoxins and MSCRAMM produced by Australian *S. aureus* of human origin.

[3]. Determination of the biofilm-forming potential of Australian versus accredited international human *S. aureus* isolates *in vitro*.

[4]. Evaluation of the efficacies of treatment of *S. aureus* biofilm *in vitro* with a biofilm degradating enzyme(s) and a broad spectrum antibiotic.

[5]. Proteomic analysis of *S. aureus* as biofilm(s) versus planktonic cultures for identification of novel biofilm-associated antigen(s).

[6]. Determine the immunogenicity and comparative protective potential of the predominant biofilm-forming antigen identified in [5].

1.6 Outline of thesis

The chapters in this thesis are assembled using a hybridization of accepted publications, submitted and/or prepared manuscripts, with individual chapters on the review of literature, general methodology not covered in the manuscripts and/or publications, discussion and references.

CHAPTER 1 and **2** – Literature Review and Materials and Methods

CHAPTER 3

Antibiotic resistance persistence was examined in biofilm vs planktonic bacteria as well as the role of capsular polysaccharide in biofilm formation. In this study, we reported a correlation between biofilm formation and the presence of PNAG however there was not an absolute correlation indicating participation of additional biofilm adhesins. This study also proves no correlation between encapsulation and the formation of biofilm in *S. aureus*. Persistence of antibiotic resistance was observed in 30 day old subcultures prepared from *S. aureus* biofilms. However, once dispersed into planktonic form, susceptibility was reverted after 4 weeks post-subculturing.

CHAPTER 4

Serological and genotyping methods were used to detect the presence of capsular polysaccharide and the surface-associated polysaccharide type 336. The 4 major types of capsule were observed between clinical and student *S. aureus* isolates used in this thesis. In this study, we observed 6 strains that were non-tyeable by serological and genotyping methods. However 4 out of the 6 were determined to possess capsule using an improved Maneval's staining method. This implicates the presence of additional capsular types among non-typeable isolates of *S. aureus*.

CHAPTER 5

In this chapter, diversity and distribution of important virulence factors of *S. aureus* among clinical and student *S. aureus* isolates used in this study was determined. Using PCR genotyping, 25 primer sets were used to determine the presence of MSCRAMM and exotoxins genes. Commercially available toxin typing kits were also used to determine the presence of the most common Staphylococcal enterotoxins. Random Amplified Polymorphic DNA (RAPD) methods were used to further characterize the diversity and distribution of virulence factors through cluster analysis. The data suggested the distribution of virulence factors are not genotype specific among the test and control isolates. This study implicates MSCRAMM Spa, IsdA, IsdB, SdrD and SdrE and the toxins SEG, α-toxin and β-toxin are predominantly expressed and provide useful targets the development of cocktail vaccines.

In **CHAPTER 6**, biofilm disruption was tested with 8 treatment groups. The biofilm disruption enzymes – DNase and Dispersin B – were tested in combination with Tobramycin, a broad spectrum antibiotic. Scanning electron microscopy was implemented to provide physical evidence of biofilm disruption and bacterial damage with a combination therapy. Dispersin B, previously thought a weak biofilm disrupter in *S. aureus* as compared to DNase, proved to be more successful with Tobramycin than DNase and Tobramycin *in vitro*. The combination of DNase and an antibiotic has previously been explored and treatment alone with Dispersin B, however this study provides a novel report of combination therapy for biofilm-associated *S. aureus* infections.

CHAPTER 7 details the proteomics analysis performed for identification of the biofilm-associated potential virulence proteins expressed *in vitro* under iron-depletion conditions. A single protein band of interest was isolated using SDS-PAGE

and subjected to proteomics analysis where Fructose-biphosphate aldolase, or Aldolase, was identified at a virulence protein in *S. aureus*.

CHAPTER 8 – Antisera against Aldolase and MntC was raised in Balb/c mice and tested for the ability to produce an immune response by detecting IgM, IgA, IgG1 and IgG2a. Antibodies were then administered to determine the potential ability as a passive immunotherapy vaccine candidate. Blood, spleen and liver were examined to determine bacterial load after a 4hr challenge. Antibodies raised were tested against the PEG method developed in **Chapter 9**.

CHAPTER 9 describes a novel biofilm inhibition assay developed using the MBEC (minimum biofilm eradication concentration) biofilm peg lid and antibodies towards several virulence factors. This new method allows for direct determination of the presence of antigen to allow or inhibit the bacteria to form biofilm on the peg lid. Predominant virulence factors identified in **Chapter 5** were tested in the biofilm inhibition assay.

CHAPTER 10 – Conclusion

1.7 References

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Chapter 2 – Generic Materials and Methods

2.1 Bacterial strains and Controls

Nineteen isolates were obtained from the Freeze-dry Microbiology database, maintained by the Microbiology Department of the School of Biomedical Sciences, Curtin University, by Mr Alain Delhaize. All the freeze-dried isolates were obtained from Royal Perth Hospital and/or Queen Elizabeth II Hospital, Perth, Western Australia. Twelve (12) strains were isolated from the undergraduate Medical Microbiology students following ethics approval by Curtin University's Human Ethics Committee, Approval Number SoBS 04/11. The clinical and student isolates used in this study are listed in Table 2.1. American Type Culture Collection (ATCC) controls were purchased as quality control strains for biofilm production and and the typing of the isolates by PCR (Table 2.2). Capsular polysaccharide control strains (Table 2.3) were kindly donated by Professor Gerald Pier, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School.

Primary identifications tests were performed all on isolates for the confirmation of *S. aureus* before storage and preservation. These include gram staining (GPC, grapevine clusters) and a positive slide coagulase test (clumping in presence of plasma). A tube coagulase was performed if there were weak slide coagulase reactions. Other tests carried out included DNase test and growth characteristics on Mannitol Salt Agar (Kateete *et al.*, 2010).

Table 2.1: List of bacterial isolates used in the project

No	Bacteria	Project ID	Source/Reference
1	*Staphylococcus aureus*	HuAISRF-Saur1	Freeze dry Microdb*
2	*Staphylococcus aureus*	HuAISRF-Saur2	Freeze dry Microdb
3	*Staphylococcus aureus*	HuAISRF-Saur3	Freeze dry Microdb
4	*Staphylococcus aureus*	HuAISRF-Saur4	Freeze dry Microdb
5	*Staphylococcus aureus*	HuAISRF-Saur5	Freeze dry Microdb
6	*Staphylococcus aureus*	HuAISRF-Saur6	Freeze dry Microdb
7	*Staphylococcus aureus*	HuAISRF-Saur7	Freeze dry Microdb
8	*Staphylococcus aureus*	HuAISRF-Saur9	Freeze dry Microdb
9	*Staphylococcus aureus*	HuAISRF-Saur11	Freeze dry Microdb
10	*Staphylococcus aureus*	HuAISRF-Saur12	Freeze dry Microdb
11	*Staphylococcus aureus*	HuAISRF-Saur13	Freeze dry Microdb
12	*Staphylococcus aureus*	HuAISRF-Saur14	Freeze dry Microdb
13	*Staphylococcus aureus*	HuAISRF-Saur15	Freeze dry Microdb
14	*Staphylococcus aureus*	HuAISRF-Saur16	Freeze dry Microdb
15	*Staphylococcus aureus*	HuAISRF-Saur18	Freeze dry Microdb
16	*Staphylococcus aureus*	HuAISRF-Saur19	Freeze dry Microdb
17	*Staphylococcus aureus*	HuAISRF-Saur20	Freeze dry Microdb
18	*Staphylococcus aureus*	HuAISRF-Saur21	Freeze dry Microdb
19	*Staphylococcus aureus*	HuAISRF-Saur23	Freeze dry Microdb
20	*Staphylococcus aureus*	H1-769199	Student strain
21	*Staphylococcus aureus*	H2-FH	Student strain
22	*Staphylococcus aureus*	H3-718972	Student strain
23	*Staphylococcus aureus*	H4-Paul C (ear)	Student strain
24	*Staphylococcus aureus*	H5-13188622 (nose)	Student strain
25	*Staphylococcus aureus*	H6-Maria	Student strain
26	*Staphylococcus aureus*	H7-MelO	Student strain
27	*Staphylococcus aureus*	H8-28062009	Student strain
28	*Staphylococcus aureus*	H9-080989	Student strain
29	*Staphylococcus aureus*	H10-38911557	Student strain
30	*Staphylococcus aureus*	H11-9555100 (nose)	Student strain
31	*Staphylococcus aureus*	H12-13965121 (nose)	Student strain

* Microbiology database (Microdb)

Table 2.2: List of bacterial isolates used as controls

ATCC ID	Strain	Clinical data	Genotype/Phenotype
ATCC® 29213™	*Staphylococcus aureus* subsp. aureus Rosenbach	Wound	Quality control strain Strong biofilm producer*
ATCC® 13565™	*Staphylococcus aureus* subsp. aureus Rosenbach	Ham involved in food poisoning	Produces large amounts of β-hemolysin*
ATCC® 49775™	*Staphylococcus aureus* subsp. aureus Rosenbach	Patient with chronic furunculosis	Produces PVL Produces γ-hemolysin*
ATCC® 51651™	*Staphylococcus aureus* subsp. aureus Rosenbach	Patient with menstrual toxic shock syndrome	Produces SEF Produces TSST-1*
ATCC® 8096™	*Staphylococcus aureus* subsp. aureus Rosenbach	Furuncle	Produces α-hemolysin*
ATCC® 55804™	*Staphylococcus aureus* subsp. aureus Rosenbach	Urine	Serotype 336*

* Information retrieved from the ATCC website

Table 2.3: Capsular polysaccharide controls

Strain ID	CP type	Source/Reference
Strain M	Type 1, or CP1	Kindly provided by Professor Gerald Pier Department of Medicine Brigham and Women's Hospital, Channing Labs, Harvard Medical School Boston, MA
Smith Diffuse	Type 2, or CP2	
Strain Newman	Type 5, or CP5	
USA 400 MW2	Type 8, or CP8	
LAC USA 300	Negative control, or CP negative	

2.2 Nutritional Media used for growth and storage of Staphylococcal cultures

The media used for the cultivation and storage of *S. aureus* and *S. epidermidis* were as follows.

2.2.1 Nutrient Broth and Agar

13g of dehydrated culture media (Oxoid) was added to 1L of distilled water. Broth base was evenly mixed and placed for sterilization autoclaving at 121°C for 30 minutes. Filter sterilized glucose solution was added to Nutrient broth to make a 1% concentration for all biofilm growth. To 100mL of Nutrient broth, 1.5g of agar was added. Solution was gently mixed and placed for sterilization 121°C for 30 minutes after which it was cooled to 56°C in a water bath. In a biohazard cabinet, plates were aseptically poured at approximately 5 to 6 plates per 100mLs.

2.2.2 Brain Heart Infusion Broth and Agar

37g of dehydrated Brain heart infusion base (Oxoid) was added to 1L of dH_20 and heated till base dissolved. Broth was placed for sterilization autoclaving at 121°C for 30 minutes. To make BHIA, 1.5g of agar was added to 100mL of BHIA and placed for sterilization 121°C for 30 minutes after which it was cooled to 56°C in a water bath. All plates were aseptically poured in a biohazard cabinet and stored at 4°C till usage.

2.2.3 Long-term storage of isolates

After primary *S. aureus* identification, cryobeads (Blackaby Diagnostics) were used for long-term storage of strains. A single swab was used to collect a thick inoculum of bacteria from a Mueller Hinton purity plate (PathWest Media) and transferred to inoculate collection tube with fluid containing cryobeads. The beads were inverted 3 times and a slim glass pasteur pipette was used to collect all remaining fluid which

was correctly discarded. The remaining beads, containing bacteria, were stored at -80°C till required. To obtain bacteria from a cryobead, a single bead was aseptically collected and placed in Nutrient broth. Broth cultures were placed at 37°C on a 120rpm orbital shaker to obtain sufficient growth.

2.3 Biofilm formation

Strains to be tested were grown overnight from a fresh cryobead in sterile nutrient broth on an orbital shaker (80rpm). Cells were pelleted at 15,000rpm for 2mins and washed twice in 1xPBS. Suspensions were adjusted to 0.132 OD_{600nm} and made into a 2% inoculum using sterile nutrient broth supplemented with 1% glucose. To a clean 96 well microtiter plate, 200 µL of the broth culture was aliquoted and placed for 18hrs at 37°C on an orbital shaker (80rpm). The plate was removed from the orbital shaker and left to stand still at 37°C for a further 24hrs.

2.4 Isolation of bacterial DNA

UltraClean® Microbial DNA Isolation Kit (Mo Bio) was used to extract DNA from *S. aureus* isolates. A purity plate was streaked out and a single colony was inoculated in 2mL of Nutrient broth. Briefly 1.8mL of an aerated overnight culture grown at 37°C was centrifuged at 12,000rpm for 30secs. The supernatant was removed and the pellet was re-centrifuged to remove remaining liquid. DNA was then extracted from the pelleted cells as per manufacturer's instruction. All extracts were quantified using the Nanodrop to ensure adequate amount of DNA was recovered after which they were stored at -20ºC until required.

2.5 Genotyping

Amplification of target genes was performed using the materials and methods as follows.

2.5.1 Preparation of primer and polymerase chain reaction

Oligonucleotides were designed using Primer-BLAST and/or Primer3Plus obtained from sequences deposited on Genbank, NCBI. Primer pairs were analysed using Primer3 to eliminate potential of self-complementary or secondary formation and rate oligonucleotides for G-C content, size, molecular weight and required Tms.

Oligonucleotides used in this project were synthesized by Geneworks with lyophilized stocks made to a stock concentration of 100µM. The final working concentration was prepared to 10pmol using RNase/DNnase-free, molecular biology grade water (Fermentas). Primer stocks and working concentrations were stored at -20°C and thawed prior to use. PCR master mixes and reactions were stored on ice during experiment preparation. Unless otherwise stated, all genomic PCR components were purchased from Thermo Scientific Fermentas unless otherwise stated. All PCR reactions were performed to amplify targeted gene sequences using the following protocol before amplification using Veriti Thermal Cyclers.

The general PCR components in the master mix are shown below.

Table 2.4: Component preparation with desired primers for gene amplification

PCR Reaction Components	Volume
PCR Master Mix (2x), Fermentas	5µL
Forward Primer, 10pmol	0.2µL
Reverse Primer, 10pmol	0.2µL
Template DNA	1 µL
Water, nuclease-free	3.6µL
Total volume	10µL

The Tm for each primer set was determined by an initial temperature gradient. PCR and primer optimization conditions were dependent on sequences and appropriate positive controls listed in their respective experiments. From the 10µL total volume, 5 - 6µL of PCR product was combined with 2µL loading buffer (30% glycerol,

0.25% bromophenol blue) and loaded on a 1.5% agarose gel in 1x Sodium Borate buffer for gel electrophoresis. Agarose preparation and components of SB stock are listed in Table 2.5

Table 2.5: Gel electrophoresis components and methods

1.5% Agarose	50x SB Buffer
1.5g agarose in 100mL 1x SB Buffer	20 g NaOH
0.8µL/100mL Midori Green stain	120 g H3BO3 (boric acid powder)
Microwaved at high for 2min, poured and set at room temperature	Bring to 1 L with dH_2O
	Adjusted to 1x SB Buffer for gel runs

Gels were run at 120V to 140V at room temperature and visualised on a UV transilluminator to observe amplified bands. Unless otherwise indicated, O'RangeRuler 100 to 1500bp DNA Ladder (Fermentas) was run to identify rough band size.

2.6 Measurement of antibody response

Measurement of isotype-specific antibody responses was carried out using indirect enzyme-linked immunosorbent assays, ELISA, which was performed according to (Chen *et al.*, 2008). The protocol used is described below.

2.6.1 Indirect ELISA

100µL of antigen comprising intact *S. aureus* was added to the allocated wells (≈ 0.250 OD_{600}nm) in PBS to the indicated wells. The microtiter plate was covered with a clear sealing film and incubated at 37°C for 2hrs or overnight at 4°C. The antigen was carefully aspirated and washed 3x with wash buffer (1x PBS with 0.05% Tween 20) with flick to remove fluid and inverting the tray on an absorbent paper towel. Approximately 100µl of blocking buffer was dispensed into the well and

incubated at 37°C for 2hrs or overnight at 4°C. Wells were washed 3x with wash buffer using the same flick and blot method. Serial doubling dilutions (100µL volume) of the primary antibody was prepared using PBS as the solvent starting from the first well (approximately 1 in 100 or 1 in 200) up to the end 7th well. The tray was incubated at 37°C for 2hrs or overnight at 4°C. A 1 in 1,000 dilution was prepared of the appropriate enzyme conjugated secondary antibody (IgG-Alkaline Phosphatase or ALP). Of this, 100µL was dispensed and incubated at 37°C for 2hrs or overnight at 4°C. The tray was washed 3x with wash buffer using the flick and blot method. To each well, 100 µl of enzyme substrate (p-nitrophenyl phosphate for ALP) was added and incubated with the tray covered in aluminium foil at room temperature for approximately 12-20 minute. Colour intensity was read at 405nm using the plate reader.

The mean absorbance of the controls was deducted from the absorbance values for each individual well. The values were plotted against the reciprocal of each dilution to produce an antibody titre deemed appropriate for further analysis.

2.6.2 Materials used in ELISA

Buffers described in the ELISA protocol are listed below with their components and preparation procedure.

Table 2.7: Buffer preparation and their components used for ELISA

Coating Buffer (g/L)	Blocking Buffer (g/L)	Substrate Buffer (g/L)
Na_2CO_3 1.56g $NaHCO_3$ 2.94g Made up to 800ml with dH2O Adjusted pH to 9.6 with 1M NaOH and then made up to 1L with d.H2O	Tris(0.01M) 1.21g NaCl (0.15M) 8.5g Gelatin (0.2%) 2.0g BSA(1%) 10g Dissolved in 750ml dH2O and made up to 1L.	Diethanolamine 106g $MgSO_4$.7H2O 0.249g Add to 800ml d.H2O Adjusted pH to 10 and then made up to 1L with d.H2O

2.7 Immunoproteomic analysis

A detailed process for the preparation of *S. aureus* protein lysates and isolation of the unique biofilm-associated antigen, fructose-1,6-bisphosphate aldolase, is described in **Chapter 7** and **8**.

2.7.1 Protein quantification

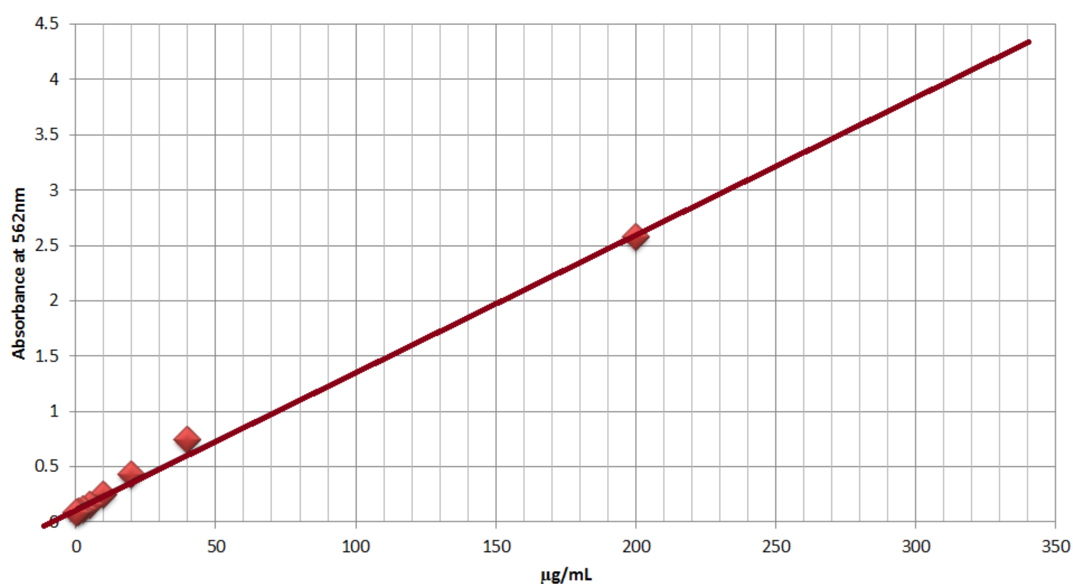
Measurement of protein concentration of lysates was determined using the Micro BCA Assay (Thermofisher). Preparation of standards and working reagents were performed according to manufactures' instructions. In a 96 well microtitre plate, total amount of 150µL of protein standard was mixed well with 150µL of working reagent. This was incubated for 2hrs at 37ºC to allow colour development. Given the following absorbance at OD562nm and their corresponding concentrations, a standard curve was obtained for a basic protein estimation profile.

Table 2.8: Protein standards and absorbance's used to plot a standard curve.

Sample	OD_{562nm}	Final BSA Concentration
A	2.581	200 µg/mL
B	0.742	40 µg/mL
C	0.431	20 µg/mL
D	0.250	10 µg/mL
E	0.160	5 µg/mL
F	0.120	2.5 µg/mL
G	0.092	1 µg/mL
H	0.090	0.5 µg/mL
I	0.000	0 µg/mL = Blank

The average absorbance of the blank was subtracted from each OD reading of the standards and unknown samples. Growth curve used in this study (Fig 2) allowed for measurement and protein quantification of samples.

Fig 2: Micro BCA Assay for protein estimation at OD_{562nm}



Note: The Micro BCA Assay was used for general estimation and compared to protein quantification determined at OD_{280nm}.

2.8 Separation of proteins on SDS PAGE

Following protein quantification, protein lysates were run on Sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE, for separation of proteins. SDS-PAGE analysis used a combination of hancast gels using the BioRad system and pre cast Novex® NuPAGE® SDS-PAGE gel system.

2.8.1 Preparation of Hand-cast gels and SDS Buffers

Buffers were stored at room temperature after preparation as detailed in Table 2.9 and Table 2.10.

Table 2.9 Buffers used in protein gel construction

4x Stacking Buffer	4x Resolving Buffer	10% Ammonium persulfate (APS)
Tris (0.5M) 30.25g pH 6.8 with HCL SDS (0.4%) 2g Made to 500mL with dH2O	Tris (15M) 91g pH 8.8 with HCL SDS (0.4%) 2g Made to 500mL with dH2O	APS 1g Dissolved in 10mLs dH20

The 10% APS solution was continuously stored at 4ºC and removed prior to gel construction. SDS buffer was prepared as a 10X running buffer store and diluted to 1x Running buffer prior to a protein gel run.

Table 2.10 SDS Buffer components

Buffer components	Concentration	10x Running Buffer
Glycine	192mM	288g
Tris	25mM	60.4g
SDS	0.1%	20g
Ingredients combined and made to 2L of dH_2O		

2.8.2 Gel compositions and PAGE method

PROTEAN® II XL Cell system by Biorad was utilized to run large SDS-PAGE for proteomic analysis. Depending on the gel percentage required (Table 2.11), handcast gels were constructed using 3mm clean glass plate (200mm x 200mm, 220mm x 200mm) with appropriate spacers and sandwich clamps.

Table 2.11 Resolving and Stacking gel compositions

Resolving Gel (20mL)	8%	10%	20%	Stacking Gel (10mL)	3%	4.5%
4x Resolving Buffer	5mL	5mL	5 mL	4x Stacking Buffer	2.5mL	2.5mL
Water	9.7mL	8.3mL	1.7 mL	Water	6.5mL	6mL
30% Acrylamide/Bis 29:1	5.3mL	6.7mL	13.3 mL	30% Acrylamide/Bis 29:1	1mL	1.5mL
TEMED	0.02mL	0.02mL	0.02mL	TEMED	0.02mL	0.02mL
10% APS	0.2mL	0.2ml	0.2 mL	10% APS	0.1mL	0.1mL

The plate sandwich was placed in a slab gel casting stand, carefully tightened and checked for leaks. Resolving gel was made up with the addition of 10% APS and Tetramethylethylenediamine (TEMED - last). Sterile dDW was placed over the resolving gel while gel polymerization occurred. After roughly 20 mins, dDW was removed and the stacking gel was poured and appropriate gel combs were inserted.

After solidification, comb was gently removed and the constructed gels were locked in placed to run in the PROTEAN® II XL Cell tank.

Approximately 350mL of SDS 1x running buffer was added to the upper tank and 1.2L to the lower tank. Tank was cooled at 10ºC constant for the duration of the gel run with water circulating pump. Samples were run overnight at 6-8mA constant using a PowerPac™ power supply (Biorad).

Assembled cassettes were removed from running tank the next morning. The protein gels were disassembled from the cassette and rinsed 3 times in dDW for 5 mins each on an orbital shaker, 80rpm.

2.8.3 Staining solutions

Coomassie staining was performed for visualization of proteins following SDS-PAGE. Gels were fixed and stained using the solutions detailed in Table 2.12. All stains were stored at room temperature.

Table 2.12 Composition of staining solutions for protein gels

Fixing Solution	Staining Solution	Destain Solution
40% Methanol 10% Acetic Acid	0.02% Coomassie R-250 30% Methanol, 10% Acetic Acid	8% Acetic Acid

Gels were fixed by the addition of fixing solution and microwaving the gel for 45 sec at high. Fixed gels were placed at room temperature on an 80rpm orbital shaker for 15 to 30 mins. The fixing step was repeated before decanting the fixing solution and adding staining solution. The gel was then microwaved at high for 45 sec. The gels were placed at room temperature on an 80rpm orbital shaker for 30 mins. After decanting the staining solution, destain solution was added and microwaved at high for 45 sec. The gel was placed on the orbital shaker until the desired background

was obtained. A change in destain solution may have been done once or twice depending on the level of background clearance.

2.8.4 Western blotting

After protein isolation from SDS-PAGE, western blot was performed on the protein of interest. Pre cast Novex® NuPAGE® SDS-PAGE gel system were (Invitrogen, Life Technologies) used for western blotting in this study. All gels were run using the XCell SureLock™ transfer system (Life Technologies) as per manufacturer's instructions. All buffers were freshly made prior to use and stored at room temperature except for Blocking buffer which was stored at 4 ºC (Table 2.13).

After running SDS-PAGE, the pre cast gel was placed in 1xTransfer buffer for 10 to 15mins prior to assemble of the XCell SureLock™ transfer cassette.

Table 2.13 Western Blot buffer compositions

Transfer Buffer		Tris Buffered Saline with Tween20 (TBST)		Blocking Buffer	
Tris (25mM)	3.03g	Tris (20nm) pH 7.5	2.42g	BSA (3%)	3g
Glycine (190nm)	14.27g	NaCl (150nm)	8.76g	TBST	100mL
Methanol (20%)	200mL	Tween20 (0.1%)	1mL	Stored at 4ºC	
Made up to 1L (pH 8.3)		Made up to 1L			

For each transfer, a nitrocellulose membrane cut to the size of the gel was pre-soaked in 1x transfer buffer. A transfer sandwich was assembled in the following order from top to bottom: blotting pad, filter paper, transfer membrane, protein gel, filter paper and blotting pad. The assemble sandwich cassette was in turn placed into the XCell

SureLock™ tank and locked in place. The inner cassette was filled with 1x transfer buffer and the outer tank with chilled dDW. Transfer was run at 30V constant for 1hr.

For protein transfer detection following antibody incubation, the Immun-Star™ AP Chemiluminescence Kit (Biorad) was implemented as a detection system before visualization on the ChemiDoc™ MP System (Biorad) imager.

2.9 Affinity Gel Chromatography

HIS-Select Nickel Affinity Gel (Sigma-Aldrich) was used for selective purification of recombinant protein, Dispersin B, which was developed to contain a histidine tag. Storage of the affinity gel (Sigma Aldrich) in 30% ethanol was delivered by the manufacturer, which was removed prior to packing into a column. The ethanol was removed by rinsing the gel contents in 1 to 2 volumes of dDW followed by equilibration with 5 volumes of equilibration buffer. The ingredients used for this process are shown below.

Table 2.14 Composition of the buffers (made up in dH_2O) using in column chromatography

Extraction Buffer	Equilibration and Wash buffer	Elution Buffer
20mM Tris-HCL pH 7.5 500mM NaCl	50 mM sodium phosphate pH 8.0 0.3 M sodium chloride 10 mM imidazole	50 mM sodium phosphate pH 8.0 0.3 M sodium chloride 250 mM imidazole

The column was packed with the washed gel by gently pouring 15mL of the packing contents into an appropriate sized column (~20mL). After column gel settling, the column was washed with 3 bed volumes of extraction buffer followed by passing cleared lysate through the column. The column was then washed with 3 bed volumes

of extraction buffer containing 5mM imidazole followed by 3 bed volumes of extraction buffer containing 20mM imidazole. The final elution of the protein was performed with wash of 2 bed volumes of extraction buffer containing100mM imidazole. Flow rate of the fraction was approximately 0.5mL/min.

Used column were washed with 4 bed volumes of wash buffer and gel contents were removed and stored in 30% ethanol at 4ºC for future usage.

2.10 Electro-transformation of pDispersin B into E. coli

Escherichia coli DH5α was transformed with the plasmid pDispersin applying standard procedures (Sambrook, 2001). The plasmid consisted of the expression vector pASK-IBA33plus (3250bp, IBA Lifesciences) containing a synthetic gene encoding dispersin B as described by Gokcen et al. (2013). Briefly, 5µl of provided pDispersin DNA was mixed gently with 40µl MAX Efficiency® DH5α™ Competent Cells (Life Technologies) and stored on ice for 30 mins.

Using 1mm electroporation cuvettes (BTX Harvard Apparatus), plasmid DNA was electroporated into MAX Efficiency® DH5α™ (1.8 v 25 uF and 200 ohms) followed by recovery on ice. Cells were placed in tubes containing LB broth and incubated at 37ºC on an orbital shaker (120 rpm) for 2 hour before being plated on LB agar containing 150µg/ml ampicillin.

2.11 Plasmid isolation and size verification

Plasmid DNA was isolated using the AxyPrep Plasmid MiniPrep Kit (Axygen Biosciences) according to the manufacturer's instructions. The plasmid preparations were checked for purity using agarose gel electrophoresis. Five microliters of plasmid DNA was loaded with 1µl of 5x loading buffer (Fermentas) onto a 1% agarose gel made up with 1x Sodium Borate buffer (10 mM NaOH, pH 8.5 with H3BO3), and electrophoresed at 100V. Gels were stained with 0.8uL/100mL Midori Green DNA Stain (Nippon Genetics) and visualized using a UV transilluminator.

The size of the isolated plasmids was verified by double restriction enzyme digest using HindIII and XbaI (Promega) according to the manufacturer's instructions. O'GeneRuler 1 kb Plus DNA Ladder (ThermoScientific) was used as a base pair marker.

2.12 Determination of DNase concentration

Staphylococcus aureus biofilms were developed as previously described. Three treatment groups were used viz., 2 concentrations of DNAse (50KU and 140KU) and one PBS treatment control group. One hundred microliters (100µL) of DNase at different concentrations was added to the allocated wells containing biofilm and placed on an orbital shaker (50rpm) for 2hrs. Biofilm cells were washed once in PBS and stained for 5mins with 0.1% crystal violet before washing twice with PBS. Crystal violet was solubilized in 96% methanol and the absorbance read at OD_{630nm}. The OD value obtained for the negative control was subtracted from those of the treatment groups.

2.13 Determination of Dispersin B activity against biofilm

Strong biofilms were developed over 3 days. Briefly, biofilms were washed twice in PBS and incubated for 2hrs at 37ºC on an orbital shaker, 80rpm with various concentration of purified Dispersin B –720µg/ml, 500µg/mL, 100µg/mL, 50µg/mL, 10µg/ml and control, µg/ml. The supernatant was removed and stained with 0.2% Crystal Violet (Sigma) after which the wells washed and air dried. Biofilm bound bacteria were solubilized in 96% Methanol and the OD obtained by reading the plate values at 630nm using the EnSpire Multimode Plate Reader (PerkinElmer).

2.14 SDS removal from purified protein(s)

Removal of SDS from purified protein(s) was performed prior to immunization in a mouse model. This was done by implementing the ProteoSpinTM Detergent Clean-

Up Micro kit (Norgen Biotek Corp) as per manufacturer's instructions. Briefly the pH of the protein sample was adjusted to 4.5 using the acidic binding buffer provided. Purified protein was then loaded onto an assembled micro spin column after column resin was prewashed with 500µL modified column activation and wash buffer (0.5mL Acidic Binding buffer, 12.5mL isopropanol, 12mL sterile deionized water). Maximum of 650µL of purified protein was loaded onto the column at one time and spun at 15,000rpm for 5mins. The process was repeated until entire sample was applied to column. After repeat washing with 250µL modified column activation and wash buffer, the column was washed with 250µL of regular column activation and wash buffer. Protein was then eluted in 2 rounds of centrifugation with 25uL of elution buffer (50mM sodium phosphate pH 12.5). Prior to elution, 5µL of neutralizer was added to the elution fraction tube to neutralize the protein elution. Proteins were quantified using the Micro BCA Assay and also quantified at OD_{280nm}. Eluted proteins were stored at -20ºC until required.

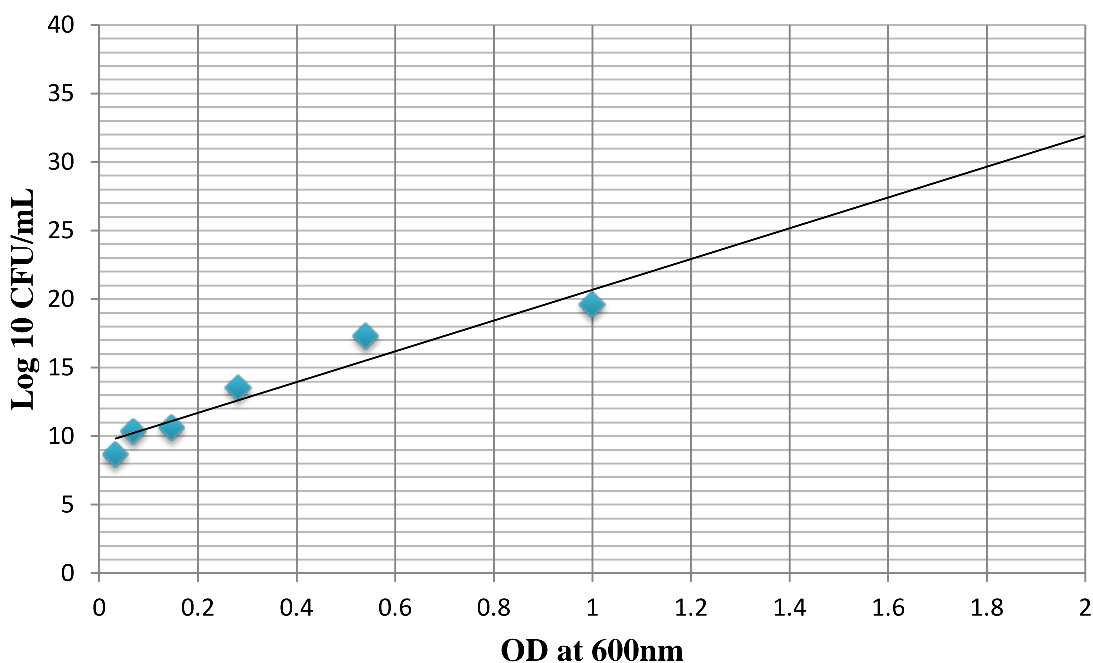
2.15 Establishment of a Growth Curve for *S. aureus*

A single colony was used to inoculate 5mL of Nutrient broth. The broth was left to grow overnight at 37ºC on an orbital shaker, 80rpm. Cells were pelleted and washed twice in cold 1x PBS with adjustment of OD_{600nm}. Serial two fold dilutions were performed in cold 1x PBS and their ODs were measured (Table 2.15). Colony forming units of each dilution was performed with 10-fold dilutions and plated out on MH plates. The plates were incubated overnight at 37ºC.

Table 2.15 Absorbance at OD_{600nm} and CFU/mLs of undiluted and doubling dilutions of *S. aureus* used for construction of the growth curve

Dilution	OD_{600nm}	CFU/mL	log10
Neat	1	3.64×10^{19}	19.5611
1 in 2	0.541	2.08×10^{17}	17.31806
1 in 4	0.283	3.2×10^{13}	13.50515
1 in 8	0.148	4.00×10^{10}	10.60206
1 in 16	0.0695	2.26×10^{10}	10.35411
1 in 32	0.0325	4.60×10^{8}	8.662758

Fig 3. Growth curve of *S. aureus* used in this study



Prior to an experiment, approximate ODs were adjusted and CFU/mL determined by plated out on MH plates with 10-fold dilutions.

2.16 References

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Chapter 3

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An original reprint of this publication is available in the Appendix

Human methicillin-sensitive *Staphylococcus aureus* biofilms: Potential associations with antibiotic resistance persistence and surface polysaccharide antigens

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Running Title: Human MSSA: Properties of Biofilm Formation

Key Words: *Staphylococcus aureus* Biofilm, Antibiotic Resistance Persistence, Polysaccharide Intercellular Adhesin, *ica* typing, Capsular Phenotype

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ABSTRACT

The development of persistent antibiotic resistance by human MSSA strains and substantial association with poly-N-acetyl glucosamine (PNAG) in biofilms is reported in this investigation. Sixteen of 31 MSSA strains under study were found to have developed resistance to one or more antibiotics, with 4 strains, 2 of which did not produce biofilms, showing resistance to cefoxitin, undetectable by *mecA* amplification. Antibiotic resistance displayed by 13/14 biofilm-forming *S aureus* isolates remained persistent for 4 weeks prior to reverting back to the original antibiotic susceptibility, prompting a suggestion of determining antibiograms for clinical *S aureus* isolates subcultured from biofilms developed *in vitro* as well as planktonic subcultures prepared from the site of infection. While there was correlation of antibiotic resistance with biofilm formation, as also demonstrated previously in other investigations, this is the first time that association of persistence of antibiotic resistance with biofilm formation is being reported. We also observed no association between biofilm formation and major capsule types. However, substantial, although not absolute, association of biofilm formation with PNAG was observed, warranting continued identification of additional surface-associated polysaccharide and/or protein antigens associated with biofilm formation for development of an effective vaccine against *S aureus* infections regardless of capsular phenotype.

INTRODUCTION

Staphylococcus aureus is a pathogenic gram-positive bacterium that has emerged as a frequent cause of nosocomial or hospital acquired infections [1]. The pathogen can cause a variety of infections ranging from superficial skin, deep seated skin, wound sepsis, pneumonia, septic arthritis, post-surgical toxic shock syndrome, endocarditis and osteomyelitis to name a few [1 ,2, 3, 4]. In a hospital setting, patients who have been surgically treated with indwelling devices or catheters have a higher rate of *S aureus* infections [5]. There has been an increasing trend in

resistance towards β-lactam antibiotics which gives rise to a severe health issue in hospital and community settings [6]. Many nosocomial *S aureus* strains have been shown to be resistant to methicillin (MRSA) [5], spread of which, associated with both nosocomial and community-acquired infections (CA-MRSA), has been reported in all continents [7]. Resistance of this bacterium to antibiotics leads to difficulty in successfully treating invasive and non-invasive *S aureus* infections. In the United States, high incidence of invasive MRSA infections have been observed, with death in about 20% of all infections, as compared to other pathogenic strains [6], with increasing incidence also observed in the UK and Australia [1, 8].

Persistence of *S aureus* in infections is dependent on a multiplicity of virulence factors promoting establishment of infection and invasion, and evading the host immune responses [6]. One of the most important virulence factors is the ability of this organism to form biofilms [1]. Biofilm or polysaccharide slime [9] has a major impact on medical implants as it increases bacterial tolerance towards antimicrobial agents and penetration of host defence elements [10]. Importantly, MRSA strains that form biofilms also develop resistance to all the commonly used antibiotics to which the planktonic bacteria are susceptible [1]. The aims of this study were to a) determine antibiotic susceptibility profile of MSSA strains isolated from biofilms versus planktonic cultures which required selection of a reproducible method for assessment of biofilm formation, and b) determine potential association of biofilm formation by MSSA with the 2 major surface-associated polysaccharides viz., polysaccharide intercellular adhesin (poly-N-acetyl glucosamine [PNAG)] and the predominant capsular types 5 or 8.

MATERIAL AND METHODS

Collection of human *S aureus* isolates

Nineteen isolates were kindly donated by the Microbiology Section, School of Biomedical Sciences, Curtin University and 12 strains were isolated from undergraduate students studying medical microbiology following approval by Curtin University's Human Ethics Committee (Approval Number SoBS 04/11). All isolates were stored on cryobeads (Blackaby Diagnostics) at -80°C for further usage.

Biofilm analysis

a) **TCP method**

This method was adapted from a procedure carried out according to Patterson *et al.* (2010) [11]. The bacterial strains were grown in a 96 well microtitre plate with nutrient broth in 37^0C orbital shaker (80 rpm) for 24hrs. The suspensions were adjusted to 10^8 cfu/mL. Two hundred and fifty microliters (250µL) of each suspension was added to a 96 well flat bottom microtitre plate and incubated at 37^0C for 18hrs on an orbital shaker after which they were removed from the shaker and left at 37^0C without shaking for the remaining 6hrs. After incubation, cells were washed with sterile saline (three times) and fixed in 96% pure ethanol. Wells were then stained with 2% crystal violet and washed three times with sterile distilled water to remove excess stain. 200uL of 33% glacial acetic acid was then added to each well and absorbance (OD) measured at 600nm. The average OD of negative control was subtracted from test values. An accredited strong biofilm producer *S aureus* ATCC29213 was also included in this study. The arbitrary cut off point used for biofilm formation was 0.120 OD_{600nm} according to Christensen *et al.* (1985) [12]. *S aureus* strains showing 4 X OD_{600nm} at the cut off point (equivalent to an OD of 0.480) or less OD were considered to represent weakly adherent biofilm forming populations, up to 6 X OD_{600nm} at the cut off point (equivalent to 0.720_{600nm}) as moderately

adherent biofilm forming populations and values greater than 6 X OD_{600nm} as strongly adherent biofilm forming populations.

b) Congo Red Agar Method

Congo Red agar plates were made as described elsewhere [13]. Briefly, plates were inoculated and placed in a 37°C hot room and observed over 72hrs for slime production. A positive result was indicated by the production of black colonies. Weak slime producers were indicated by red/pink growth [13]. This experiment was repeated three times to ensure reproducibility. Accredited strong biofilm producer *S aureus* ATCC29213 was also included in this investigation.

Antibiotic Sensitivity/Susceptibility Testing Method

For a comparison between free planktonic and biofilm-associated bacteria, antibiotic sensitivity plates (PathWest) were inoculated using the CDS method [14]. Briefly, bacteria were grown in 2mL of nutrient broth supplemented with 2% glucose. Broths were left in 35°C for 48hrs to allow adequate biofilm development, after which the supernatant was removed. Bacteria grown in biofilm and free-floating bacteria were streaked for single colonies on MH plates (PathWest). Single colony for each was stabbed with a straight wire, suspended in 2.5mL saline and flooded onto Sensitest plates (PathWest). Plates were dried for 15 min in 37°C hot room after which the following antibiotics discs (Oxoid) were carefully placed on each plate: benzylpenicillin 0.5ug (P 0.5), cefoxitin 10ug (FOX 10), cephalexin 100ug (CL 100), ciprofloxacin 2.5ug (CIP 2.5), co-trimoxazole 25ug (SXT 25), erythromycin 5ug (E 5), linezolid 10ug (LZD 10), mupirocin 200ug (MUP 200), rifampicin 1ug (RD 1), teicoplanin 15ug (TEC 15), tetracycline 10ug (TE 10) and vancomycin 5ug (VA 5). Zones of inhibition 6 mm or greater were recorded as sensitive except VA5 and TEC15 where zones greater than or equal to 2 mm were recorded as sensitive.

DNA extraction

Using an extraction kit (MO-Bio), all 31 the *S aureus* strains were placed for DNA extraction. All extracts were stored at -20°C until required for experimentation after which they were thawed and placed on ice.

Capsular polysaccharide (CP), icaA/D and mecA typing

DNA extracts of the 31 *S aureus* isolates were subjected to PCR for CP types 5 or 8, *ica* A/D and *mecA* gene expression as follows:

(a) Capsular polysaccharide typing

For CP typing, primers published by Moore and Lindsay (2001) [2] were used (CP5 forward 5'-ATGACGATGAGGATAGCG-3' and CP5 reverse 5'-CTCGGATAACACCTGTTGC-3'; and CP8 forward 5'-ATGACGATGAGGATAGCG-3' and reverse 5'-CACCTAACATAAGGCAAG-3'). Predicted product sizes and Tm were 880 and 1147 bp, and 60°C and 53°C, for CP5 and CP8 respectively. PCR cycling condition were 95°C for 5mins, 95°C for 30sec, Tm for 30secs, 72°C for 5min (x25) and extension at 72°C for 5 min. PCR product was electrophoresed in 1xTAE buffer in a 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen).

b) ica typing

DNA extracts of the 31 *S aureus* isolates were run against *ica*A and *ica*D primers published by Vasudevan *et al.* (2003) [15]. The primers used for *ica*A and *ica*D typing were *ica*A forward was 5'- CCTAAC TAACGAAAG GTAG-3', *ica*A reverse 5'- AAGATATAGCGA TAAGTG C-3'; and *icaD* forward 5'-AAACGTAAGAGAGGTGG-3'and *ica*D reverse 5'-GGCAATATGATCAAGATAC-3' respectively. Predicted band size for *ica*A was 1315bp with a Tm of 48°C and predicted band for *ica*D was 381bp with a Tm of 47°C. PCR run cycle was 95°C for 5mins, 95°C for 45secs, Tm for

45secs, 72°C for 5mins (x30) and extension at 72°C for 5 mins. PCR product was run in 1xTAE buffer in a 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen).

c) *mecA* typing

Detection of the *mec*A gene was carried out as described previously [16] using the following primers: *mec*A forward 5'-AAAATCGATGGTAAAGGTTGGC-3' and *mec*A reverse 5'-AGTTCTGCAGTACCGGATTTGC-3'. Predicted band size was 533bp with a Tm of 52°C. The PCR was run on a cycle of 94°C for 5mins, 95°C for 30secs, Tm for 30secs, 72°C for 60secs (x25) and extension at 72°C for 10 mins. The PCR product was then electrophoresed in 1xTAE Buffer on a 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen).

RESULTS

Using the TCP method, 31 strains were assessed for biofilm production including one strong biofilm producing ATCC *S aureus* strain 29213. This method revealed that all human *S aureus* isolates were biofilm producers with 14 (45.2%), 15 (48.4%) and 2 (6.4%) strains showing strong, moderate and weak biofilms respectively (Table 1). Using the CRA method, colonies that are red or dark red in colour indicate negative biofilm production. Colonies that stained black were labelled as biofilm producers. Out of 31 strains including ATCC, 12 (38.7%) samples were positive with black colonies (biofilm producers) whereas 19 (61.3%) were negative for biofilm production with red colonies after 72hrs in a 37°C. The ATCC *S aureus* strain 29213, an accredited strong biofilm producer, also displayed dark black colonies as anticipated. It was thus clear that the TCP method was better than the CRA method for detection of biofilm producers despite the observation of varying degrees of biofilm formation (Table 1).

Table 1. Typing of human *S aureus* isolates

Strain Number	CP5	CP8	*icaA*	*icaD*	CRA	TCP
SA 1	−	−	−	+	−	+/−
SA 2	−	+	−	+	−	+
SA 3	+	−	−	+	+	+
SA 4	−	+	+	+	+	+
SA 5	−	−	+	−	+	+
SA 6	+	−	−	+	+	+
SA 7	+	−	+	+	−	+
SA 9	+	−	+	+	+	++
SA 11	−	+	+	+	−	+
SA 12	+	−	+	+	−	+/−
SA 13	−	+	+	+	−	++
SA 14	−	+	+	+	−	+
SA 15	−	+	+	+	−	+/−
SA16	−	+	+	+	−	++
SA 18	+	−	+	+	+	++
SA 19	+	−	+	+	−	++
SA 20	+	−	+	+	−	+
SA 21	+	−	+	+	−	++
SA 23	−	−	+	+	−	+
SA-H1	−	−	−	−	−	+
SA-H2	−	−	−	−	−	+
SA H3	−	+	+	+	+	++
SA H4	−	+	+	+	+	+
SA H5	−	−	−	−	+	++
SA H6	+	−	+	+	−	++
SA H7	+	−	+	+	+	++
SA H8	−	+	+	+	−	+
SA H9	−	+	+	+	+	++
SA H10	−	+	+	+	+	++
SA H11	−	+	+	+	−	++
SA H12	−	+	+	+	−	+
SA ATCC29213	+	−	+	+	+	+

*For CP typing, Ica typing and CRA, results listed as Negative (−) and positive (+)
*For TCP method, results listed as Negative (−), weak (+/−), moderate (+) and strong positive (++)

Using the CP typing method, 11/31 strains were CP5 positive (35.5%), 15/31 were CP8 positive (48.4%) with 5 strains being untypeable (16.1%) (Table 1). Twenty-three (23) of the 31 strains used in this study revealed possession of both *ica*A and *ica*D genes, which were either CP5 or CP8 positive. Nine (9) of the 23 *ica*A *ica*D

positive strains were moderate biofilm producers whereas 13/23 strains were strong biofilm producers. Out of the remaining 9 strains, one strain was positive for either *ica*A with the remaining 4 strains being positive for the *ica*D gene only. Three *S aureus* strains that were all CP negative were also *ica*A and *ica*D negative (Table 1).

Antibiotic sensitivity tests on the planktonic cultures of human *S. aureus* isolates revealed that they were all methicillin sensitive (MSSA) with similar results being obtained *mec*A gene typing (data not shown). However, when assessed for antibiotic susceptibility of *S aureus* isolated from the biofilms, 16 of these isolates had developed resistance towards TE 10, TEC 15, P 0.5, CIP 2.5, SXT 25, CL 100 and FOX 10 upon cultivation as biofilms (Table 2).

Table 2. Development of antibiotic resistance in MSSA strains in biofilms

Antibiotic ↓, Strain →

	SA1	SA3	SA4	SA6	SA7	SA9	SA12	SA16
TE 10	S→R	R	S	R	S→R	S	S→R	R
RD 1	R	R	S	R	R	S	R	S
TEC 15	S→R	S	S→R	S	S	S	S	S
FOX 10	S	S→R	S	S	S→R	S	S	S
P 0.5	S→R	S	S	S	R	S	S	R
E 5	S	R	S	S	R	S	S	S
CIP 2.5	S	S	S	S→R	S	S	S	S
SXT 25	S	S	S	S	S	S→R	S	S
MUP 200	S	S	S	S	S	S	S	S
LZD 10	S	S	S	S	S	S	S	S
CL 100	S	S	S	S→R	S→R	S	S	S→R
VA 5	S	S	S	S	S	S	S	S

	SA19	SA23	H2	H6	H8	H9	H10	H11
TE 10	S→R	R	S	R	S	S	S	S
RD 1	S	S	S	S	S	S	S	S
TEC 15	S	S→R	S	S	S	S	S	S
FOX 10	S	S	S→R	S→R	S	S	S	S
P 0.5	R	R	R	R	R	S→R	S→R	S→R
E 5	S	S	S	S	S	S	S	S
CIP 2.5	S	S	S	S	S→R	S	S	S
SXT 25	S	S	R	S	S	S	S	S
MUP 200	S	S	S	S	S	S	S	S
LZD 10	S	S	S	S	S	S	S	S
CL 100	S	S	S	S	S	S	S	S
VA 5	S	S	S	S	S	S	S	S

S → R: Sensitive to resistant, S: Sensitive, R: Resistant

It was thus clear that a high rate of resistance to antibiotics developed when MSSA isolates were grown as biofilms. To determine the persistence of antibiotic resistance, *S aureus* from biofilms were subcultured for 30 days and their antibiotic resistance profile determined at day 30 when it was discovered that the resistance to most antibiotics was maintained (Table 3) as judged by the fact the persistence of antibiotic resistance by 13/14 moderate to strong biofilm forming *S aureus* strains.

Table 3. Antibiotic resistance profile of *S. aureus* strains after 30 days of subculturing.

Antibiotics	Antibiotic resistance of human *S. aureus* strains	
	Resistance at Day 1	Resistance at Day 30
TE 10	SA1, SA3, SA6, SA7, S12 SA16, SA19, SA23, H6	SA1, SA3, SA6, SA7, SA16, SA19, SA23, H6
TEC 15	SA1, SA4, SA23	SA1, SA23
P 0.5	SA1, SA7, SA16, SA19, SA23 H2, H6, H8, H 9, H10, H11	SA1, SA7, SA16, SA19, SA23 H2, H6, H8, H 9, H10
CIP 2.5	SA6, H8	H8
SXT 25	SA9	SA9
CL 100	SA6, SA7, SA16	SA7, SA16
FOX 10	SA3, SA7, H2, H6	SA3, SA7, H2, H6

SA denotes *S. aureus*

Of the 16 strains that developed antibiotic resistance, 14 strains were either strong or moderate biofilm producers whereas two strains were weak biofilm formers indicating an excellent correlation between antibiotic resistance and biofilm production. On the other hand, 11/31 of human *S. aureus* biofilm producing isolates were encapsulated indicating a lack of correlation of the capsule with biofilm formation. On the other hand, 23/31 MSSA strains that were *ica*A *ica*D positive (74%) were biofilm producers indicating a substantial but not absolute correlation with biofilm formation/production.

Eleven of the 16 antibiotic resistant strains, 11 strains (68.75%) *S aureus* possessed both *ica*A and *ica*D genes, essential for production of PNAG [17], a potential contributor to biofilm formation, indicating a substantial relationship with antibiotic resistance. Three of the 16 antibiotic resistant strains were CP negative, 7 CP5

positive and 6 CP8 positive indicating a lack of correlation of antibiotic resistance with encapsulation. One *ica* negative strain (SA-H2) that was classified as a moderate biofilm producer, developed resistance to benzylpenicillin 0.5ug (P 0.5) and cefoxitin 10ug (FOX 10). Of the 4 cefoxitin resistant strains, one was CP negative while the other three were CP5 positive. All four strains were found to have retained their resistance to cefoxitin after 4 weeks of biofilm formation (Table 3).

DISCUSSION

The resistance of microbial biofilms towards antimicrobial reagents has been the subject of intense interest and yet little is known about the mechanisms of involved. Mah *et al.* (2001) [10] have suggested that maturity of the biofilm is a function of slow growth, stress response and quorum sensing. While biofilms of the common opportunistic pathogens are widely distributed, the resistance mechanisms operating in biofilm formation appear to be distinct from those responsible for conventional antibiotic resistance. However, studies have also shown that biofilm bacteria that were once resistant can revert to sensitivity upon dispersion of the biofilm [18].

Formation of biofilm is regulated by a single *icaADBC* operon, which produces the proteins IcaA, IcaD, IcaB and IcaC. These proteins are involved in the production of the polysaccharide intercellular adhesion, poly-β-1,6-linked *N*-acetylglucosamine or PNAG, the major exopolysaccharide in the *S aureus* biofilm matrix. The expression of *ica*A and *ica*D genes is of utmost importance in the activation of PNAG synthesis [17]. PNAG and is structurally and functionally similar to polysaccharide intercellular adhesion or PIA which is produced by *Staphylococcus epidermidis* [19]. PNAG is considered to be one of the key components of the cell surface that mediates bacterial adherence to host surfaces, enabling biofilm formation and protection [20]. Another component that enables *S aureus* to resist host defence systems is the production of a capsular polysaccharide or CP. It is generally observed that bacteria that possess an extracellular CP are the “culprit” for invasive diseases [21]. This CP enables the bacteria to evade the host immune response by

resisting phagocytosis. The two major serotypes expressed are serotypes 5 and 8 that account for approximately 25% to 50% of human isolates, respectively [21].

Staphylococci, in particular *S aureus*, are frequent pathogens in hospital and community acquired settings [1]. This pathogen has emerged as a chronically infecting pathogen, which has demonstrated resistance to multiple antibiotics leading to strains that are methicillin resistant or MRSA [5]. In the US alone, it is estimated that up to 20% of patients undergoing surgery will acquire one or more nosocomial infections costing up to $10 billion [22]. Furthermore, the World Health Organization, (2001) [23] recently estimated that the overall prevalence of hospital-associated infections in developed countries to be between 5.1% and 11.6%, with (a) more severe a burden in neonatal care, critical care and elderly patients who lack immune function as compared to the general population, and (b) higher rate of mortality in patients who develop septicaemia and pneumonia [23]. The rate of infection in developing countries was found to be several folds higher as compared to developed countries [23].

S aureus possesses several immune evasion strategies such as production of leukocidal toxins in particular, capsular polysaccharides and Microbial Surface Components Recognizing Adhesive Matrix Molecules or MSCRAMM [24]. However, one additional characteristic of importance is the ability of *S aureus* to form biofilms at the site of infection. In this investigation, we found that even approximately 50% of *S aureus* isolates that are methicillin-sensitive (MSSA) as planktonic cultures acquired resistance to one or more antibiotics upon biofilm formation confirming previous reports [10, 18]. However, we found that the biofilm-associated acquired antibiotic resistance by the *S aureus* isolates persisted for 4 weeks when grown as planktonic cultures, representing a matter of serious concern in the therapy of staphylococcal infections. While there was an association between biofilm formation and antibiotic resistance developed by MSSA strains as was apparent with all the 16 biofilm-producing *S aureus* strains, this association was not absolute with the persistence of the acquired antibiotic resistance warranting further investigations. It was interesting, however, that none of the MSSA strains used in

this study, that acquired resistance to cefoxitin (4/16) were originally resistant to cefoxitin, the antibiotic used in many pathology laboratories for determination of susceptibility to methicillin [25], with the remaining 12/16 (75%) strains displaying resistance to one or more other antibiotics.

Notwithstanding the suggestion of using more than one method for assessment of biofilm formation of *S aureus*, the presented data prompts a recommendation that antibiotic susceptibilities of clinical *S aureus* isolates be determined from cultures of biofilm-associated *S aureus* developed *in vitro*, in addition to the planktonic cultures prepared directly from the infection site, for optimal therapeutic outcomes particularly for stubborn hospital and community acquired staphylococcal infections including those associated with biomaterial implants [26].

Although there was a general trend of development of antibiotic resistance in *S. aureus* strains expressing both *ica*A and *ica*D genes, 75% (12/16) antibiotic resistant strains showing this trait, the correlation was not absolute The absence of 100% correlation of PIA/PNAG of *S aureus* with biofilm formation is not surprising given the reported participation of other virulence antigens in biofilm formation such as fibronectin-binding proteins, FnBPA and FnBPB [28], collagen-binding adhesion (cna) proteins and clumping factor (clfA) [29].

In summary, our findings suggest that there is no correlation between biofilm formation and encapsulation but there is substantial, although not absolute, association with PNAG confirms the need to continue identification and characterization of other polysaccharide and non-polysaccharide MSCRAMM participating in biofilm formation as is actively being pursued in some laboratories [4, 28, 29]. Our data also suggests that serious consideration should be given to determining antibiograms for *S. aureus* isolated from patients using both biofilms developed *in vitro* as well as planktonic cultures prepared from specimens taken directly from the site of infection for achievement of potentially better therapeutic outcomes.

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Chapter 4

Waryah CB, Gogoi-Tiwari J, Wells K, Costantino P, Al-Salami H, Sunagar R, Isloor S, Hegde N, Richmond P & Mukkur T (2014) Serological versus molecular typing of surface-associated immune evading polysaccharide antigens - based phenotypes of *Staphylococcus aureus*. J Med Microbiol, Nov 63(Pt 11):1427-31. Impact factor of 2.266

An original reprint of this publication is available in the Appendix

Serological versus molecular typing of surface-associated immune evading polysaccharide antigens – based phenotypes of *Staphylococcus aureus*

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Running title: Surface-associated polysaccharide antigens of *S. aureus*

Key Words: Capsular polysaccharides, Polysaccharide antigen 336, *Staphylococcus aureus*, Serological typing, Genotyping, Capsular staining

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ABSTRACT

Aim of this study was to compare the performance of serological versus molecular typing methods to detect capsular polysaccharide (CP) and surface-associated polysaccharide antigen 336 phenotypes of *Staphylococcus aureus* isolates. Molecular typing of CP types 1, 5 and 8 was carried out using PCR whereas serological typing of CP1, 2, 5, 8 and antigen 336 was carried out by slide agglutination using specific antisera. By genotyping, 14/31 strains were CP8 positive, 12/31 strains were CP5 and the remaining 6/31 isolates were non-typeable (NT). One isolate was positive for both CP5 and CP8 by PCR but was confirmed as CP8 type serologically. Detection of CP2 and type 336 by PCR was not possible because specific primers were either not available or were non-specific. Using serotyping, 14/31 strains were CP8 positive, 11/31 CP5 positive and 2/31 positive for antigen 336. The remaining four *S. aureus* isolates were serologically NT. However, three of 4 NT and two 336-positive *S. aureus* isolates were encapsulated as determined by light microscopy after capsular staining. This discovery was surprising and warrants further investigations on the identification and characterisation of additional capsular phenotypes prevalent among *S. aureus* clinical isolates. It was concluded that serological typing was a better method than molecular typing method for use in epidemiological investigations based upon the distribution of surface-associated polysaccharide antigens-based phenotypes.

INTRODUCTION

Staphylococcus aureus is an important human pathogen causing a broad range of infectious diseases facilitated by its ability to asymptomatically colonize healthy individuals (Daum & Spellberg, 2012; Foster, 2004). The most common conditions associated with this pathogen include wound infections, boils, carbuncles and impetigo, which typically follow abrasions of the skin or mucosal surfaces. The organism can further invade the body or be introduced through medical devices, resulting in systematic infections ranging from osteomyelitis and pneumonia to septicaemia, meningitis and endocarditis (O'Riordan & Lee, 2004; Tzianabos *et al.,*

2001). *Staphylococcus aureus* is also a common pathogen of immuno-compromised patients and a leading nosocomial pathogen in nursing homes, neonatal care and intensive care units (Ohlsen & Lorenz, 2010).

Staphylococcus aureus produces several virulence factors, among which the capsular polysaccharides (CP), which are anti-phagocytic (Sutter *et al.,* 2011), have been widely used as vaccine targets (O'Riordan & Lee, 2004; Robbins *et al.,* 2004). Initial studies, using agglutination tests, reported the existence of 11 CP types based on serological specificity (Sompolinsky *et al.,* 1985; Karakawa et al., 1988). However, studies carried out later reported the existence of only four capsular types, 1, 2, 5 and 8, with the remaining types representing mutated forms of one or more of the CP types (O'Riordan & Lee, 2004, Fattom *et al.,* 1998). Many previous studies reported majority of human *S. aureus* strains (70-80%) to possess either CP5 and/ or CP8 (Skurnik *et al.,* 2010; Roghman *et al.,* 2005; Verdier *et al.,* 2007), which underpinned the rationale of targeting these two predominant types for the development of conjugate vaccines against infections caused by *S. aureus*. *Staphylococcus aureus* strains that harboured the capsule locus for CP5 or CP8, but were non-typeable by serological methods for CP1, 2, 5, and 8, were labelled as serotype 336, a surface-associated polysaccharide antigen that is a variant of *S. aureus* cell wall teichoic acid (Sutter *et al.,* 2011).

The aim of this study was to compare the performance of serological versus molecular typing methods in determining the distribution of different surface-associated capsular and the somatic polysaccharide 336 phenotypes of *S. aureus* isolated from Western Australians.

METHODS

Collection of isolates

A total of 31 *S. aureus* isolates were used in this investigation. Nineteen of these isolates were obtained from Royal Perth Hospital and Queen Elizabeth II Hospital in Perth, Western Australia and 12 isolates collected from undergraduate laboratory medicine students in the School of Biomedical Sciences, Curtin University, Perth, Western Australia (Human Ethics approval Number SoBS 04/11). Positive controls used in this investigation were *S. aureus* Strain M (CP1), Smith Diffuse (CP2), Strain Newman (CP5), USA 400 (CP8), LAC USA 300 (CP negative) and an antigen 336 positive ATCC *S. aureus* strain, 55804.

DNA extraction

Prior to use, the strains were freshly cultured in nutrient broth (PathWest media) with a cryobead followed by incubation overnight in a shaking incubator at 37°C. DNA was extracted using a commercial kit (MO-Bio) and stored at -20°C. All extracts were thawed on ice prior to genotyping using PCR.

Genotyping of CP types

The PCR primers (Geneworks) used in this study are shown in Table 1. PCR parameters for cap1 and cap2 were as follows: 94°C for 5min (initial denaturation), then 25 cycles of 94°C for 30sec (denaturation), Tm for 30sec (annealing) and 72°C for 60sec (extension) and 72°C for 5min (final extension). PCR parameters for cap5 and cap8 were the same as described previously (Babra *et al.,* 2013).

Table 1: PCR primers used for capsular polysaccharide typing

Target gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Tm	Expected size (bp)	Reference
cap1	AGG TCT GCT AAT TAG TGC AA	GAA CCC AGT ACA GGT ATC ACC A	570C	550	(Gogoi-Tiwari et al, unpublished)
cap2	AGC AAT CTT CGG TTA TTG CCG GTG	ATG ACG GTA AGG CAT CAA GGT CG	600C	731	(Gogoi-Tiwari et al, unpublished)
cap5	ATG ACG ATG AGG ATA GCG	CTC GGA TAA CAC CTG TTG C	540C	881	(12)
cap8	ATG ACG ATG AGG ATA GCG	CAC CTA ACA TAA GGC AAG	520C	1148	(12)

PCR products were separated on a 1.5% agarose gel in 1xTAE buffer and the gel stained with Midori Green 0.8µL/100mL (Nippon Genetics). The positive controls used for the PCR were Strain M (CP1), Smith Diffuse (CP2), Strain Newman (CP5), USA 400 (CP8) and the negative control, LAC USA 300.

CP Serotyping

Serotyping was carried out using an agglutination test as described elsewhere (Verdier *et al.,* 2007). CP-specific antisera were raised in specific pathogen-free Quackenbush mice, against CP1, CP2, CP5, CP8 and antigen 336 as described according to Gogoi-Tiwari et al (unpublished). Briefly, mice were immunized with *S. aureus* strains M (CP1), Smith Diffuse (CP2), Newman (CP5), USA MW2 (CP8), USA LAC 300 (CP-negative) and ATCC 55804 (336) using the following immunization schedule: The first three doses were administered at days 0, 7, 14 and 21. Each dose (0.2 mL per mouse subcutaneous) consisted of formalin-killed *S. aureus* without an adjuvant ($5x10^7$ CFU, $1x10^8$ CFU and $5x10^8$ at days 0, 7, 14 and 21, respectively). The 4th and 5th doses contained $1x10^9$ CFU and $5x10^9$ CFU, respectively, mixed equally with the Imject Alum Adjuvant (Thermo Scientific).

Non-specific reactivity of the typing sera was eliminated by cross-absorption with appropriate *S. aureus* cells of different serotypes including the accredited antigen 336-strain (ATCC 55804).

Microscopic detection of capsules

The capsules were stained using a modified Maneval's method (Maneval, 1941; Engelkirk & Duben-Engelkirk, 2008). Briefly, the modified method involved scraping of biofilm-associated cells, which were spun down at 6,000 rpm for 2 minutes and the bacterial pellet washed once with 1xPBS. Cells were then suspended in a solution of 5% sucrose and centrifuged at 6,000rpm for 2 minutes. Supernatant was removed and the pellet was suspended once more in 5% sucrose. Cells were centrifuged at 9,000rpm for 2 minutes and supernatant was removed to obtain as much pellet as possible. Cells in the pellet was gently emulsified in a drop of 1% Congo red on a clean microscope slide and air-dried. The slide was then flooded with Muir's Mordant (also known as Maneval's stain) and left to stand for 2 minutes before rinsing gently with tap water. The slide was then blot-dried using clean filter paper and viewed using an oil immersion objective (1000x magnification).

RESULTS AND DISCUSSION

Summary of results obtained using genotyping versus serotyping methods is shown in Table 2. Both genotyping and serotyping methods revealed that none of the strains were positive for CP1. Serotyping was the only effective method for the detection of CP2-postive *S. aureus* strains because the designed primers for *cap2* were non-specific and cross-reacted with the positive control strains for *cap5, cap8* and *cap1*, producing 731bp amplicons (data not shown). However, none of the strains were found to be CP2-positive by serology. Genotyping for *cap5* identified 12/31 strains (37.5%) to be positive, while one strain produced amplicons of respective expected sizes for both *cap5* and *cap8* (Table 2). Serologically, however,

this strain agglutinated only with anti-CP8 serum. Both the genotyping and serotyping results were in agreement for CP8, where 14/31 (43.75%) of the isolates were positive. As primers for type 336 were not available, genotyping for antigen 336 could not be carried out at this time. Using PCR, 6/31 (19.35%) of the isolates were regarded as NT isolates. Two of the 6 NT strains, or 2/31 (6.25%) of the total isolates that were non-typeable either by genotyping or serotyping, were found to be antigen 336-positive by serotyping. Taken together, a total of 4/31 strains or 12.9% were regarded as being non-typeable.

Table 2: Summary of genotyping and serotyping results

Capsular type	Number (and percentage) of isolates
	Detection by PCR
CP1	0 (0%)
CP2	NA*
CP5	12** (37.5%)
CP8	14 (43.75%)
336	PNA***
NT	6 (19.35%)
	Detection by serology
CP1	0 (0%)
CP2	0 (0%)
CP5	11 (35.5%)
CP8	14 (43.75%)
336	2 (6.45%)
NT	4 (12.9%)
	Detection by staining
Positive reference strains M (CP1), Smith Diffuse (CP2), Newman (CP5) and MW2 (CP8)	Capsule visible on all the strains
Negative reference strain US LAC 300	No capsule visible
CP5 and CP8 seropositive isolates	Capsule visible on 26 out of 26
Non-typeable	No capsule visible on 1 out of 4 Capsule visible on 3 out of 4
336 positive	Capsule visible on 2 out of 2

*Not applicable – the primers produced non-specific bands with positive controls for *cap*1, *cap*5 and *cap*8 besides *cap*2; **Includes one strain that showed positive for both CP5 and CP8 by genotyping however confirmed to be CP8 by serotyping; ***Primers not available

All of the strains were then subjected to capsular staining. The bacterial cell stained red/purple against a dark background with the capsules appearing as unstained white

halos. Strain USA LAC 300 (CP negative) and one of our test isolates, H7, which was positive for CP5 by genotyping (Babra *et al.,* 2013) and serotyping (this study), were used as negative and positive controls respectively (Figures 1 and 2). All of the *cap8* positive isolates were found to have a capsule as did all the *cap5* positive isolates including one strain that was positive for both CP5 and CP8 by PCR but was CP8 positive by serology. Quite surprisingly, it was discovered that 3 of the 4 NT isolates were also encapsulated when subjected to capsular staining (Figure 3).

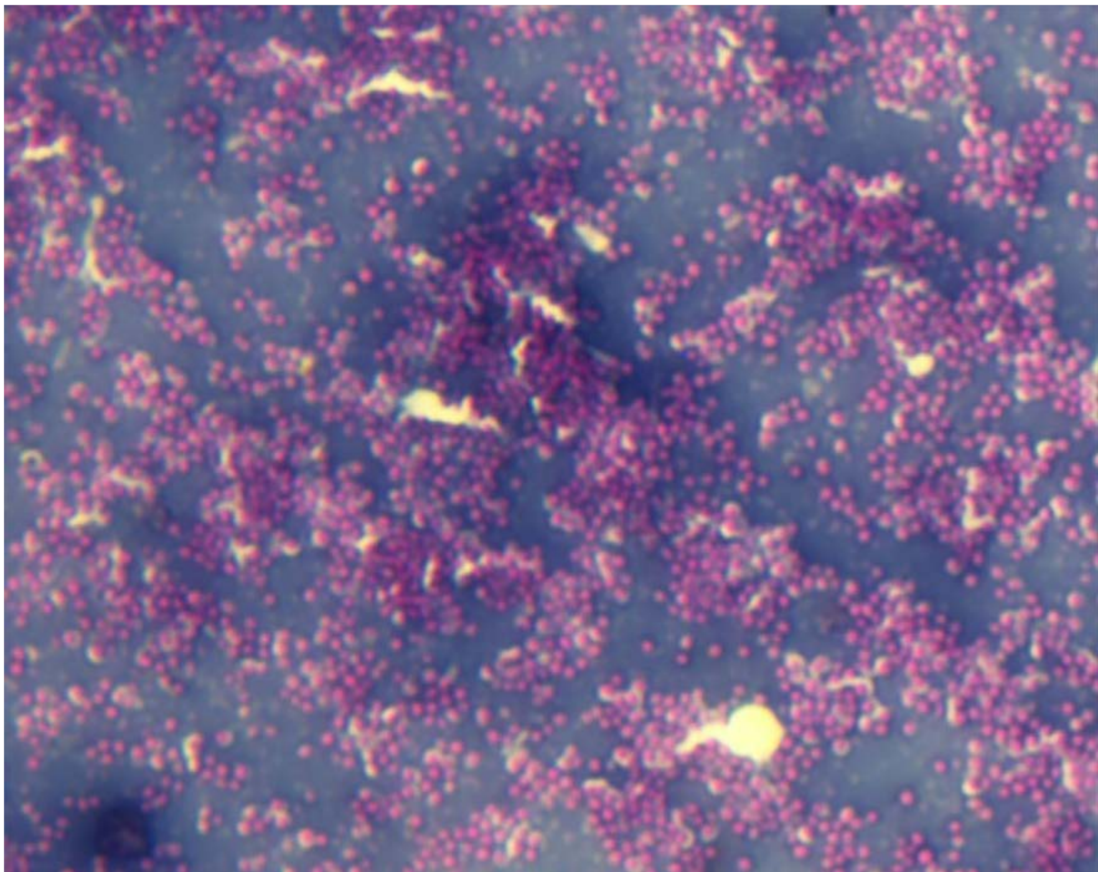

Fig 1: Negative control (USA LAC 300) *S. aureus* isolate stained with modified Maneval's staining method (1000 x magnification).

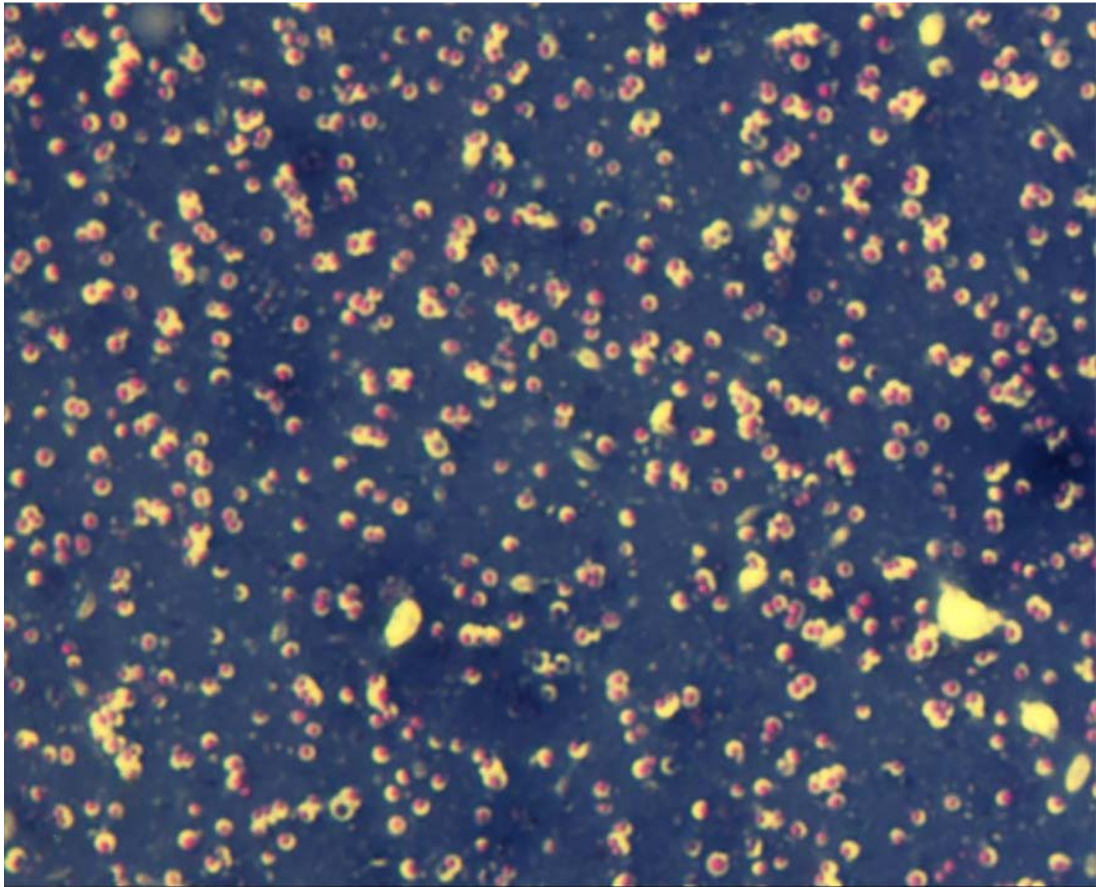

Fig 2: Positive CP control *S. aureus* isolate (H7) stained with modified Maneval's capsule staining method (x1000 magnification).

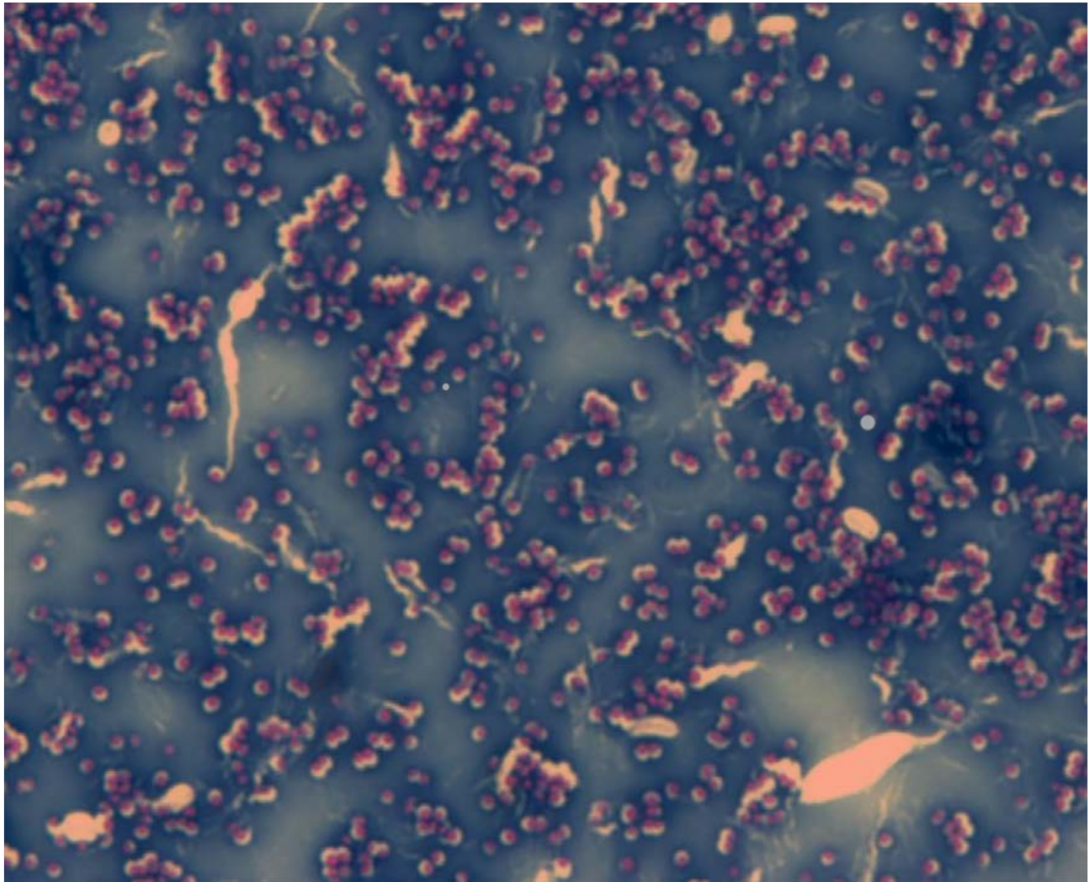

Fig 3: A non-typeable CP isolate of *S. aureus* strain negative (by genotyping and serotyping) displays a capsule using modified Maneval's capsule staining method (x1000 magnification).

Staphylococcus aureus is the cause of multiple disease syndromes in both community and hospital settings. A well-known and established key factor in its virulence is the production of a capsule (Englekirk & Duben-Engelkirk, 2008), an important immune evasion molecule of *S. aureus* (Nanra *et al.*, 2012). As such it has been used as a target for vaccine development and evaluated as a key component of conjugate vaccines in pre-clinical models as well as in human trials (Nanra *et al.,* 2012; Pozzi *et al.,* 2012).

Our study has shown that capsular phenotypes 5 and 8 were the predominant capsular phenotypes among the Western Australian *S. aureus* isolates included in this investigation. However, we found that serological typing using slide agglutination was better for determining capsular phenotype than the genotyping method because of the lack of availability of specific primers for detection of CP2 and antigen 336. Serologically, 81.5% of the total *S. aureus* isolates were comprised of CP8 (43.74%) and CP5 (37.5%), confirming previous reports from select other countries (Roghmann *et al.,* 2005; Verdier *et al.,* 2007; Skurnik *et al.,* 2010), the remaining isolates being either antigen 336-positive or non-typeable.

Sompolinsky et al. (1985) performed capsular typing of *S. aureus* isolated from human infections for the 11 capsular serotypes by precipitation and agglutination with specific antisera. This research group reported that 63% of their isolates were type 8, 16% were type 5, 2% were type 7 and 0.3% were type 10, with more than 90% of total isolates being encapsulated. The remaining 10% of the encapsulated isolates were not identified as belonging to the 11 known capsular types, which may represent a prototype capsule that is different from the accepted 11 serotypes (Sompolinsky *et al.,* 1985). This is in contrast to the previous report that the lack of expression of a capsule by NT strains was due to random point mutations in the CP5A promoter or replacement by the insertion sequence IS257 (Cocchiaro *et al.,* 2006). Our study demonstrated the existence of more serotypes than just the four capsular types [CP1, 2, 5, 8] and also raises a question on the validity of the antigen 336 as a somatic non-capsular antigen.

Given that most vaccines have employed surface-associated polysaccharide antigens particularly CP5 and CP8, conjugated with one or more potential adhesins such as alpha toxin, ClfB and IsdB (Pozzi *et al.,* 2012), coupled with the fact that no protection is expected to be imparted against infections caused by NT *S. aureus*, it is important to gain knowledge on the nature of the antigens unique to NT isolates, including new capsular antigens/phenotypes, for the formulation of an improved vaccine against *S. aureus*. Our study has highlighted the potential importance determining the prevalence of not only the major capsular serotypes, CP5 and CP8, of *S. aureus* but also of other antigens particularly antigen 336. The fact that 75% of the NT *S. aureus* strains and the antigen 336-positive strain were also encapsulated, even by light microscopy, warrants further investigations on the identification of additional capsular types present among the NT isolates for complete epidemiological investigations and formulation of appropriate conjugate vaccines.

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CONFLICT OF INTEREST: None

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Chapter 5

<u>Waryah CB,</u> Gogoi-Tiwari J, Wells K, Yui-Eto K, Masoumi E, Costantino P, Kotiw M & Mukkur T (2014) Diversity of Virulence Factors Associated with Western Australia Methicillin-Sensitive *Staphylococcus aureus* Isolates of Human Origin.

Manuscript Submitted

Diversity of Virulence Factors Associated with Western Australia Methicillin-Sensitive *Staphylococcus aureus* Isolates of Human Origin

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Running Title: Human MSSA: Diversity of Virulence Factors

Key Words: *Staphylococcus aureus* virulence, surface adhesion, toxins, RAPD-PCR, biofilm formation, toxins, MSCRAMM, genetic similarity

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ABSTRACT

An extensive array of virulence factors associated with S. aureus has contributed significantly to its success as a major nosocomial pathogen in hospitals and community causing variety of infections in affected patients. Virulence factors produced by this opportunistic pathogen include immune evading polysaccharides such as capsular polysaccharides, poly-N-acetyl glucosamine and teichoic acid in addition to damaging toxins including haemolytic toxins, enterotoxins, cytotoxins, exfoliative toxin and microbial surface-associated components recognizing adhesive matrix molecules (MSCRAMM). In this investigation, 31 West Australian S. aureus isolates of human origin and 6 controls were analyzed for relative distribution of virulence-associated genes using PCR and/or an immunoassay kit, and MSCRAMM by PCR-based typing. Genes encoding protein MSCRAMM viz., Spa, ClfA, ClfB, SdrE, SdrD, IsdA and IsdB were detected >90% of the isolates. Gene encoding α-toxin was detected in >90% of the isolates whereas genes encoding β-toxin and SEG were detectable in 50 - 60% of the isolates. Genes encoding the toxin proteins viz.,,SEA, SEB, SEC, SED, SEE, SEH, SEI, SEJ, TSST, PVL, ETA and ETB were detectable in >50% of the isolates. Use of RAPD-PCR for determining the virulence factor-based genetic relatedness among the isolates revealed five cluster groups confirming genetic diversity among the MSSA isolates.

1. Introduction

Staphylococcus aureus is a frequent opportunistic pathogen known to cause a wide variety of diseases ranging from skin infections, such as boils and carbuncles to more serious infections such as toxic shock syndrome, endocarditis, pneumonia and sepsis [1-4]. This has led to the emergence of *S. aureus* as a common cause of hospital acquired and community acquired infections [5, 6].

The pathogenesis of *S. aureus* is attributed to several virulence factors including biofilm formation and associated prolonged persistence of antibiotic resistance, and the production of a wide array of toxins [5, 7]. A biofilm or slime, defined as a

congregation of microorganisms residing in a protective extracellular matrix [8, 9], constitutes the first step in initial attachment followed by colonization and subsequent infection. Colonization is commonly associated with an assortment of adherence factors or adhesins which aid bacterial attachment to the host surface using microbial surface component recognizing adhesive matrix molecules (MSCRAMM). Over 20 different MSCRAMM, which can be expressed in *S. aureus*, have been identified [10]. Major protein adhesins in this group include biofilm-associated protein (Bap), clumping factors A and B (ClfA, ClfB), fibronectin binding proteins A and B (FnBPA, FnBPB), collagen-binding protein (Cna), bone sialoprotein binding protein (Bbp), iron regulated surface determinant A and B (IsdA, IsdB), serine aspartate repeat gene proteins D and E (SdrD, SdrE) and Protein A (Spa) [11-14]. Following adherence, the biofilm is further strengthened by an intracellular adhesin encoded by the *ica* operon (*ica*A, *ica*B, *ica*C and *ica*D genes) which produce the cell surface polysaccharide poly-N-acetyl β-1-6 glucosamine (PNAG) and another antigen 336, a derivative of cell wall teichoic acid [13, 15, 16]. A strong relationship between PNAG and biofilm formation, although not absolute, was previously reported [8, 17].

In addition to the possession of MSCRAMM, *S. aureus* also produces a range of exotoxins that aid in host tissue membrane disruption providing nutrients essential for bacterial cell growth [18, 19] with some also contributing to biofilm formation. Exotoxins produced include cytotoxins, Panton Valentine leucocidin (PVL) and haemolysins (α, β, γ), which possess the ability to form pores in host cells enabling lysis [20, 21]. Additional toxins encoded for and/or produced include toxic shock syndrome toxin (TSST-1) and the staphylococcal enterotoxins or SE (SEA-SEE, SEG-SEJ), some of which are best characterized as super-antigens in reference to their ability to activate the proliferation of T-cells leading to release of increasing levels of pro-inflammatory cytokines [22, 23]. These also include the rare and virulent exfoliative toxins ETA and ETB [24].

The increasing trend towards development of persistent antibiotic resistance improves the ability of this pathogen to resist treatment with antibiotics [5, 25] a fundamental feature in the development of chronic infections. Aim of this study was to determine the diversity of distribution of the major MSCRAMM and toxins among the West Australian *S. aureus* isolates of human origin, using serological and/or genotypic analysis and determine their genetic relatedness.

2. Materials and Methods

2.1 Collection of strains

A total of 19 human *S. aureus* strains donated by different clinical pathology laboratories to the School of Biomedical Sciences in West Australia were kindly donated by Mr Alain Delhaize, Senior Technical Manager, responsible for managing this collection. The remaining 12 *S. aureus* isolates were collected from the laboratory medicine students enrolled in Medical Microbiology (Human Ethics approval Number SoBS 04/11) and 5 accredited capsular (CP) positive or negative control strains were kindly provided by Professor Gerald Pier, Channing Laboratory, Brigham and Women's Hospital. The 5 accredited CP positive or negative control strains used in this investigation included Strain M (CP1), Smith Diffuse (CP2), Strain Newman (CP5), USA 400 (CP8) and LAC USA 300 (CP neg). The 6th control strain was ATCC® 29213™, a strong biofilm former. All strains were subjected to preliminary microbiological testing to confirm *S. aureus* [26] and methicillin-sensitivity (MSSA) as described elsewhere [5]. All *S. aureus* strains were stored at -80°C on cryobeads (Blackaby Diagnostic Pty Ltd, WA) for future studies. Positive ATCC toxin typing controls used in this study were ATCC® 13565™ for β-hemolysin, ATCC® 49775™ for PVL and γ-hemolysin, ATCC® 51651™ for TSST-1 and ATCC® 8096™ for α-hemolysin.

2.2 Bacterial strain growth

Pure colonies of *S. aureus* strains were inoculated in sterile nutrient broth dispensed in McCartney vials and incubated at 37°C for 24hrs in a shaker incubator.

2.3 DNA extraction

All strains were subjected to DNA extraction using the Mo-Bio DNA Extraction kit (MO BIO Laboratories, Inc Carlsbad, CA). All extracts were stored at −20 °C until used.

2.4 Detection of genes encoding PVL and mecA

Utilization of the GenoType® MRSA assay (Hain-Lifesciences) was used for detection of PVL and the presence of methicillin resistance. Briefly, DNA was isolated from cultured media and amplified with biotinylated primers. The amplified product was bound using a DNA strip technology that permitted visual identification of the presence of *mecA* and PVL genes in *S. aureus*.

2.5 Detection of S. aureus enterotoxins

A SET-RPLA Toxin Detection kit purchased from Themo-Fisher Scientific Australia was used to serologically type SEA, SEB, SEC and SED. Briefly, latex sensitized with a combination of anti-enterotoxin A-D types serially diluted and added to the bacterial suspension. After 24hr incubation at room temperature, each well was observed for agglutination, which indicated the presence of enterotoxins.

2.6 Genotyping of S. aureus strains

Determination of the presence of enterotoxins, mentioned in section 3.5, was further confirmed by genotyping. Because the scope of detection of the exotoxins produced by the *S. aureus* isolates was limited because of the lack of availability of serological kits, the presence of a number of other toxins, described below, was carried out by genotyping.

The primers used in this investigation with their respective melting temperature (Tm), band size and references are shown in Table 1. Briefly, the conditions used for detection of different virulence factors were as follows:

Amplification of *tsst-1, clfA, clfB, cna* and *spa* was performed at 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.
Amplification of *fnBpA, fnBpB, hlb, sdrE, bbp, isdA and sdrD* and *sdrE* genes was performed at 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. Primers for *isdB* were developed in this study and amplified with the following 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.

Amplification of *hla* genes was performed 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. While amplification of *sea, seb, sec, sed, see, seg, seh, sei* and *sej* was performed at 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, amplification of *eta* and *etb* were performed 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. Amplification of *hlb* was performed at 95°C for 5 min, 35 cycles of 95°C for 45 sec, Tm for 45 sec and 72°C for 1 min with a final extension at 72°C for 10min.

Table 1. Primers used for detection of exotoxins and MSCRAMM using conventional PCR

Proteins targeted	Primer Forward (5'-3')	Primer Reverse (5'-3')	Tm	Expected band size (bp)	Reference
Cna Collagen binding protein	AAA GCG TTG CCT AGT GGA GA	AGT GCC TTC CCA AAC CTT TT	50°C	192	[2]
ClfA Clumping factor A	CGC CGG TAA CTG GTG AAG CT	TGC TCT CAT TCT AGG CGC ACT T	55°C	314	[27]
ClfB Clumping factor B	ATG ATC TTG CTT GCG TT	CCG ATT CAA GAG TTA CAC C	47°C	215	[27]
Spa Protein A	TCA AGC ACC AAA AGA GGA AGA	GTT TAA CGA CAT GTA CTC CGT TG	51°C	Variable	[28]
FnBPA Fibronectin binding protein A	GCG GAG ATC AAA GAC AA	CCA TCT ATA GCT GTG TGG	48°C	1279	[29]
FnBPB Fibronectin binding protein B	GGA GAA GGA ATT AAG GCG	GCC GTC GCC TTG AGC GT	56°C	820	[29]
Bbp Bone sialoprotein binding protein	AAC TAC ATC TAG TAC TCA ACA ACA G	ATG TGC TTG AAT AAC ACC ATC ATC T	53°C	575	[30]
IsdA Iron regulated surface determinant A	CTG CGT CAG CTA ATG TAG GA	TGG CTC TTC AGA GAA GTC AC	52°C	332	[25]
IsdB Iron regulated surface determinant B	ACG AGA GTT TGG TGC GCT AT	GTT GAG GCC CCT ACT TCT GA	55°C	192	This study
SdrD Serine aspartate repeat gene D	CGG AGC TGG TCA AGA AGT	TGC CAT CTG CGT CTG TTG	52.3°C	500	[25]

	AT	TA			
SdrE Serine aspartate repeat gene E	AGA AAG TAT ACT GTA GGA ACT G	GAT GGT TTT GTA GTT ACA TCG T	50°C	433	[31]
TSST-1 Toxic shock syndrome toxin	ACC CCT GTT CCC TTA TCA TC	TTT TCA GTA TTT GTA ACG CC	53°C	326	[32]
ETA Exfoliative toxin A	GCA GGT GTT GAT TTA GCA TT	AGA TGT CCC TAT TTT TGC TG	58°C	93	[33]
ETB Exfoliative toxin B	ACA AGC AAA AGA ATA CAG CG	GTT TTT GGC TGC TTC TCT TG	58°C	226	[33]
Hla Alpha toxin	GTA CTA CAG ATA TTG GAA GC	GTA ATC AGA TAT TTG AGC TAC	47°C	274	[34]
Hlb Beta toxin	GCC AAA GCC GAA TCT AAG	CGC ATA TAC ATC CCA TGG C	51°C	840	[29]
SEA Staphylococcal enterotoxin A	TTG GAA ACG GTT AAA ACG AA	GAA CCT TCC CAT CAA AAA CA	50°C	120	[35]
SEB Staphylococcal enterotoxin B	TCG CAT CAA ACT GAC AAA CG	GCA GGT ACT CTA TAA GTG CC	50°C	478	[35]
SEC Staphylococcal enterotoxin C	GAC ATA AAA GCT AGG AAT TT	AAA TCG GAT TAA CAT TATA CC	50°C	257	[35]
SED Staphylococcal enterotoxin D	CTA GTT TGG TAA TAT CTC CT	TAA TGC TAT ATC TTA TAG GG	50°C	317	[35]
SEE Staphylococcal enterotoxin E	AGG TTT TTT CAC AGG TCA TCC	CTT TTT TTT CTT CGG TCA ATC	50°C	209	[35]
SEG	AAG TAG	AGA	55°C	287	[35]

Staphylococcal enterotoxin G	ACA TTT TTG GCG TTC C	ACC ATC AAA CTC GTA TAG C			
SEH Staphylococcal enterotoxin H	GTC TAT ATG GAG GTA CAA CAC T	GAC CTT TAC TTA TTT CGC TGT C	48.4°C	213	[35]
SEI Staphylococcal enterotoxin I	GGT GAT ATT GGT GTA GGT AAC	ATC CAT ATT CTT TGC CTT TAC CAG	50°C	454	[35]
SEJ Staphylococcal enterotoxin J	CAT CAG AAC TGT TGT TCC GCT AG	TGA ATT TTA CCA TCA AAG GTA C	50°C	142	[35]

All PCR products were subjected to electrophoresis on a 1.5% agarose gel and stained with 0.8uL/100mL of Midori Green DNA Stain (Nippon Genetics) in a 1x Sodium Borate Buffer (1x SB Buffer). An O'RangeRuler DNA Ladder, 100-1500 bp, (Fermentas) was used to observe approximate band sizes on the gel which was visualised on a UV transilluminator.

3.7 RAPD analysis

Three sequence primers, previously published were used for RAPD-PCR test to provide more information on clinical, student and control strains used in this study [36]. Primer C (5′-AGGGAACGAG-3′), OPA9 (5′-GGGTAACGCC-3′) and OPA13 (5′-CAGCACCCAC-3′) were used to amplify using 1 cycle of 94 °C for 60sec, 35 cycles of 94 °C for 35sec, 33 °C for 30 s, 72 °C for 65 sec, followed by 1 cycle 72 °C for 7 min [36].

All PCR products were run on a 1% agarose gel in 1xSB Buffer. Gel was stained with Midori Green and viewed under a UV transilluminator. Bacterial DNA was randomly selected to run in duplicates to ensure reproducibility of amplification. Bands were scored in binary code with a factor of 1 representing presence of band

and a factor of 0 representing absence of bands. Results of the 3 primer sets were banded to produce a dendrogram using UPMA (DenoUPMA, http://genomes.urv.cat/UPGMA/index.php) and using the Jaccard coefficient to determine the relatedness and level of similarity between the isolates used in this study.

3. Results and Discussion

Several MSCRAMM were detected by genotyping in a high percentage of *S. aureus* isolates. These included genes encoding the proteins ClfA, ClfB Spa, SdrD, SdrE, IsdA and IsdB (Table 2). On the other hand, genes encoding the Bbp, FnBpB and Cna proteins were detectable in less than 50% of the isolates, gene encoding FnBpA protein being detectable in the smallest percentage of the isolates.

The average number of MSCRAMMs detected in this study was approximately 7, with 27 strains having a range of >6-10 (data not shown). In only 4/31 strains, 5 MSCRAMM or less were detected. Compiled results for MSCRAMM typing are shown in Table 2.

Table 2. Distribution of MSCRAMM detected by genotyping

Gene encoding	Number of positive isolates (%)
SpaA	28 (90.32%)
FnBPA	2 (6.45%)
FnBPB	13 (41.93%)
Cna	12 (38.71%)
ClfA	26 (83.87%)
ClfB	27 (87.1%)
SdrD	28 (90.32%)
SdrE	30 (96.77%)
Bbp	14 (45.16%)
IsdA	28 (90.32%)
IsdB	30 (96.77%)

Among the toxins, the most prevalent toxin detected by genotyping among the *S. aureus* isolates was α-toxin, 2nd and 3rd most prevalent detected toxins being the enterotoxin G and β-toxin (Table 3). The genes encoding other toxins were prevalent in less than 30% of the isolates, lowest being the exfoliative toxins A and B. No strain was positive for genes encoding PVL toxin.

Table 3: Distribution of different toxins detected by genotyping and/or serotyping

Encoding gene	Number of positive isolates (%)
Staph Enterotoxin A	8 (25.8%)
Staph Enterotoxin B	6 (19.35%)
Staph Enterotoxin C	3 (9.68%)
Staph Enterotoxin D	0 (0%)
Staph Enterotoxin E	0 (0%)
Staph Enterotoxin G	19 (61.29%)
Staph Enterotoxin H	4 (12.9%)
Staph Enterotoxin I	9 (29.03%)
Staph Enterotoxin J	0 (0%)
Tsst-1	8 (25.8%)
PVL	0 (0%)
Alpha toxin	30 (96.77%)
Beta toxin	13 (49.93%)
Exfoliative toxin A	1 (3.23%)
Exfoliative toxin B	1 (3.23%)

Twenty-three strains possessed genes encoding 2-4 different types of toxins. Only 3 strains possessed the gene for one toxin and 5 strains expressed genes for >5 toxins. The average number of toxins produced by the *S. aureus* strains in this study was 3 toxins (data not shown).

The SET-RPLA Toxin Detection kits were able to detect fewer toxins as compared to SE genotyping (Table 4). Of the 8 SEA positive *S. aureus* strains, only 3 were detected in serotyping and of 6 SEB positive strains, only 1 was detected in serotyping (Table 4). Of the 3 SEC positive strains, only 2 were detected by

serotyping; however the genotyping and serotyping correlated with 0 positives by both methods (not significant at the $p<0.05$ level, but substantial at $p<0.06$).

Table 4: Correlation of serotyping versus genotyping methods for the major super-antigenic enterotoxins

Toxin	Serotyping (n=31)	Genotyping (n=31)	Pearson correlation coefficient r
SEA	3 (9.68%)	8 (25.8%)	0.553
SEB	1 (3.23%)	6 (19.35%)	0.371
SEC	2 (6.45%)	3 (9.7%)	0.891
SED	0 (0%)	0 (0%)	Not possible to calculate the r value but can be assumed to be 1.0

PCR typing was more sensitive than immunoassays in detecting the genes associated with toxin production.

Accredited test capsular control strains were not positive for genes encoding SED, SEE, PVL, ETA or ETB. All test control strains were positive for α, β and the TSST toxins and Spa, ClfA, ClfB, SdrE and SdrD MSCRAMM (Table 5).

Table 5: Typing of control *S. aureus* strains

Control stain	Detectable toxin genes
ATCC 29213	SEA, SEC, SEG, SEI, TSST, α-toxin, β-toxin
Strain M (CP1)	SEA, SEC, SEG, SEH, SEI, TSST, α-toxin, β-toxin
Smith Diffuse (CP2)	SEA, SEB, SEC, SEG, SEH, SEI, TSST, α-toxin, β-toxin
Strain Newman (CP5)	SEA, SEG, SEI, TSST, α-toxin, β-toxin
USA 400 MW2 (CP8)	SEA, SEC, SEG, SEH, TSST, α-toxin, β-toxin
LAC USA 300 (CP neg)	SEG, SEH, SEI, TSST, α-toxin, β-toxin
	Detectable MSCRAMM
ATCC 29213	FnBPA, Spa, ClfA, ClfB, Bbp, SdrE, SdrD, IsdA
Strain M (CP1)	FnBPA, Spa, Cna, ClfA, ClfB, SdrE, SdrD, Bbp, IsdA
Smith Diffuse (CP2)	FnBPA, FnBPB, Spa, Cna, ClfA, ClfB, SdrE, SdrD, IsdA
Strain Newman (CP5)	FnBPB, Spa, Cna, ClfA, ClfB, SdrE, SdrD, Bbp
USA 400 MW2 (CP8)	FnBPA, Spa, Cna, ClfA, ClfB, SdrE, SdrD, Bbp, IsdA
LAC USA 300 (CP neg)	FnBPA, FnBPB, Spa, Cna, ClfA, ClfB, SdrE, SdrD, Bbp

Smith Diffuse *S. aureus* (CP2) expressed 9 MCRAMMs and 9 toxins, the highest of the control strains. Strain M (CP1) expressed 9 MSCRAMM and 8 toxins, USA 400 MW2 (CP8) expressed 9 MSCRAMM and 7 toxins, LAC USA 300 (CP neg) expressed 9 MSCRAMM and 6 toxins, ATCC 29213 expressed 8 MCRAMMs and 7 toxins and Strain Newman (CP5) expressed 8 MSCRAMM and 6 toxins.

Amplification with primer OPA09 and OPA13 yielded 4 RAPD patterns from 3 distinct bands each whereas amplification with Primer C yielded 6 RAPD patterns from 4 distinct bands. Presence or absence of bands resulted in binary data that was analyzed to produce a dendrogram. Using RAPD analysis, 5 cluster groups displaying the distribution of MSCRAMM and toxins between the groups were discernible (Figure 1).

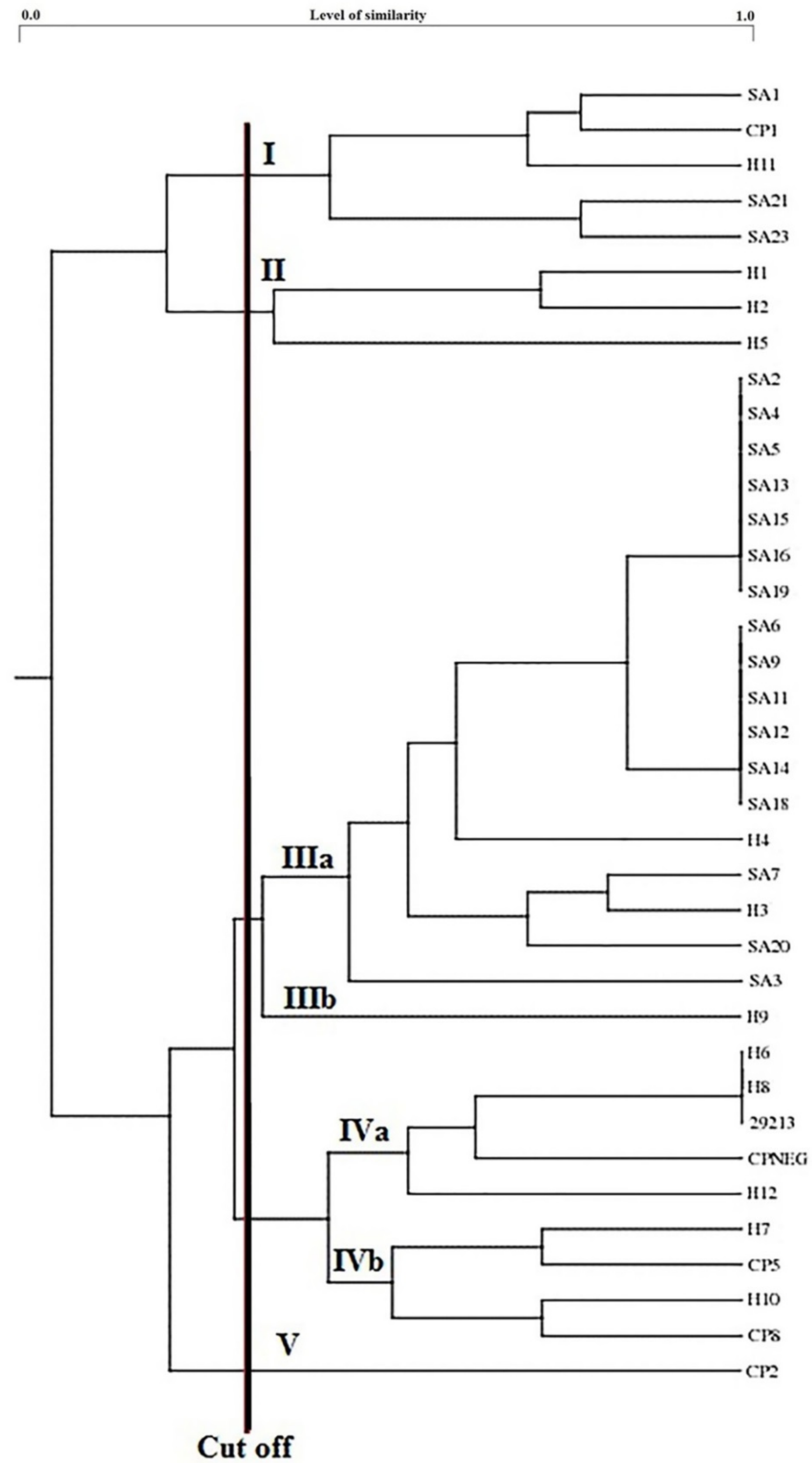

Figure 1: RAPD-based dendrogram indicating the genetic relatedness among *S. aureus* isolates including the control isolates

The cluster cut off point was determined at 33% level of similarity (0.333) resulting in 5 major cluster groups (Table 6) viz., Cluster Ia and Ib (level of similarity 0.667 to 0.800), Cluster IIa and IIb (level of similarity 0.333 to 0.750), Cluster IIIa and IIIb (level of similarity 0.333 to 1.000), Cluster IVa and IVb (level of similarity 0.500-0.600) and Cluster V (level of similarity 1.000), that were used to compare the cluster groups (Table 6).

Table 6: Distribution of the known MSCRAMM and toxins produced by the strains used in this study.

Group	Strains and subgroups (n)	MSCRAMM	Toxins
I	Group Ia (3)	FnBPA, FnBPB, Spa, Cna, ClfA, ClfB, SdrE, SdrD, Bbp, IsdA, IsdB	SEA, SEC, SEG, SEH, SEI, TSST, α-toxin, β-toxin, ETA, ETB
	Group Ib (2)	FnBPB, Spa, Can, ClfA, ClfB, SdrE, SdrD, Bbp, IsdA, IsdB	SEB, SEG, SEH, SEI, TSST, α-toxin, β-toxin
II	Group IIa (2)	FnBPa, FnBPB, SpA, ClfA, ClfB, SdrE, SdrD, IsdA, IsdB	SEB, SEG, TSST, α-toxin
	Group IIb (1)	FnBPB, SdrE, SdrD, IsdA, IsdB	SEC, α-toxin
III	Group IIIa (18)	FnBPA, FnBPb, SpA, ClfA, ClfB, Cna, Bbp, SdrE, SdrD, IsdA, IsdB	SEA, SEB, SEC, SEH, SEI, TSST, α-toxin, β-toxin
	Group IIIb (1)	Spa, Cna, ClfA, ClfB, SdrE, SdrD, IsdA, IsdB	SEC, SEG, TSST, α-toxin
IV	Group IVa (5)	FnBPA, FnBPB, Spa, ClfA, ClfB, SdrE, SdrD, Bbp, IsdA, IsdB	SEA, SEC, SEG, SEH, SEI, TSST, α-toxin, β-toxin
	Group IVb (4)	FnBPA, FnBPB, Cna, Spa, ClfA, ClfB, SdrE, SdrD, Bbp, IsdA, IsdB	SEA, SEC, SEG, SEH, SEI, TSST, α-toxin, β-toxin
V	Group V (1)	FnBPA, FnBPB, SpA, Cna, Clfa, Clfb, SdrE, SdrD, Bbp, IsdA, IsdB	SEA, SEB, Sec, SEG, SEH, SEI, TSST, α-toxin, β-toxin

It can be seen that the majority of *S. aureus* isolates were clustered into Group IIIa, with 58% (18/31) of the isolates displaying clonal similarity of MCRAMM and toxins. The genetic diversity or data on virulence factors associated with clonal complexes of MSSA strains in West Australia has not been reported unlike reports recently conducted in Europe [37]. Given that infections and persistence of *S. aureus* is a multi-step process involving several virulence factors [7, 15], the information gained in this study may assist in the development and/or formulation of vaccines that can successfully in preventing infections caused by *S. aureus* by blocking the function of the MCSCRAMM or toxins with the greatest prevalence among the *S. aureus* isolates. Further studies are clearly warranted to test this hypothesis.

The current strategies used for the development of vaccines against infections caused by *S. aureus* targeting a limited number of single antigens [15] may or may not be effective for global vaccine usage because of the differences in the distribution of genes encoding different virulence factors as demonstrated by this study. A relatively recent study demonstrated that each bacterial strain isolated from patients enrolled in their study displayed a different antibody responses triggered by 19 antigens [25].

Ideally, an effective *S. aureus* vaccine must generate protective immunity that can neutralize the major exotoxins and interfere with adhesion facilitated by the major MSCRAMM participating in biofilm formation and colonization by this pathogen. Many different types of vaccines including MSCRAMM-based vaccines [38], capsular polysaccharide and/or PNAG-based conjugate vaccines [3, 15, 38] involving conjugation of one to 3 MSCRAMM [27, 38] or selected inactivated toxins including α-toxin encoded by the *hla* gene [3, 28, 38-41] have been evaluated using passive and/or active immunization of mice. However, none of these vaccines were considered provide satisfactory protection, essentially resulting in the hope of ever developing an effective vaccine against *S. aureus* infections for use in humans [42], particularly after observing antigenic competition subsequent to co-administration of CP-based and PNAG-based conjugate vaccines [43]. Fortunately, not all the potential options for the development of an effective vaccine against infections caused by *S.*

aureus have been exhausted if one was to take the distribution of virulence antigens among the isolates in to account as an important parameter that was investigated in this study.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Chapter 6

<u>Waryah CB,</u> Wells K, Ulluwishewa D, Chen-Tan N, Gogoi-Tiwari J, Ravensdale J, Costantino P, Gökçen A, Vilcinskas A, Wiesner J, & Mukkur T (2015) Combination of different biofilm-degrading enzymes may compromise enhancement of the antimicrobial efficacy of antibiotics against *Staphylococcus aureus*.

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Combination of different biofilm-degrading enzymes may compromise enhancement of the antimicrobial efficacy of antibiotics against *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus in biofilms is highly resistant to the treatment with antibiotics, to which the planktonic cells are susceptible. This is likely to be due to the biofilm creating a protective barrier that prevents antibiotics from accessing the live pathogens buried in the biofilm. *S. aureus* biofilms consist of an extracellular matrix composed of, but not limited to, extracellular bacterial DNA (eDNA) and Poly-β-1, 6-N-acetyl-D-glucosamine (PNAG). Our study revealed that despite inferiority of dispersin B, an enzyme that degrades PNAG, to DNase I that cleaves eDNA, in dispersing the biofilm of *S. aureus*, both enzymes were equally efficient in enhancing the antibacterial efficiency of tobramycin, a model broad-spectrum antibiotic used in this investigation. However, a combination of these two biofilm-degrading enzymes was significantly less effective in enhancing the antimicrobial efficacy of tobramycin than the individual application of the enzymes. These findings indicate that combinations of different biofilm-degrading enzymes may compromise the antimicrobial efficacy of antibiotics and need to be carefully assessed *in vitro* before being used for treating medical devices or in pharmaceutical formulations.

INTRODUCTION

Bacterial adaption leading to antibiotic resistance has become the greatest challenge in the development of successful antimicrobial therapies[1]. A history of over usage of antibiotics and underestimation of the bacterial ability to adapt to the host and environment has rendered many pathogenic bacteria virtually untreatable[1]. *Staphylococcus aureus* can cause a wide range of infections in immunocompromised individuals owing to its ability to asymptomatically colonize healthy individuals as part of the normal flora[2]. Many *S. aureus* strains have acquired a remarkable resistance against β-lactam antibiotics leading to an uphill battle against infections caused by this potential pathogen[3]. Infections with community and hospital acquired methicillin resistant *S. aureus* (MRSA) have been documented worldwide[4].

Staphylococcus aureus has been associated with primary infections in patients diagnosed with cystic fibrosis and chronic ear infections, particularly in indigenous Australian populations[5], and is one of the principal causes of premature death in cystic fibrosis patients when co-infected with *Pseudomonas aeruginosa*[6, 7]. The lungs of cystic fibrosis sufferers have a high level of sputum production which impairs host clearing mechanisms, thus increasing the risk of biofilm formation by the normal flora in the lung[8].

Production of biofilms by *S. aureus* is a significant factor involved in colonization and persistence of infections[9] as it protects the bacteria from host immune defense as well as providing a protective barrier allowing for resistance to antimicrobial therapy[10]. A major structural component of *S. aureus* biofilms is represented by poly-β-1,6-N-acetyl-D-glucosamine (PNAG)[3, 14]. As a second important component, extracellular DNA (eDNA) is present in many biofilms formed by *S. aureus*[11].

Dispersin B is a 40 kDa soluble glycoside hydrolase produced by the periodontal disease-associated bacterium *Actinobacillus actinomycetemcomitans*[12, 13]. Due to its ability to specifically cleave the β-1,6-glycosidic bonds in PNAG, dispersin B efficiently degrades pre-formed biofilms of *S. epidermidis*, but is only moderately effective in degrading *S. aureus* biofilms[12, 14, 15]. There are reports of PNAG independent biofilm formation[16], however majority of *S. aureus* contain the *ica* operon cluster required for PNAG production and biofilm formation[17].

In contrast, DNase I detaches biofilms formed by *S. aureus* but displays only moderate activity on *S. epidermidis* biofilms[11]. Dispersin B was reported to sensitize *S. epidermidis*, but not *S. aureus,* biofilms to killing by the cationic detergent cetylpyridinium chloride (CPC), whereas DNase I also sensitized *S. aureus* biofilms to CPC killing, suggesting differenced in the accessibility of PNAG and eDNA in the biofilms of these organisms[15].

We hypothesized that treatment of *S. aureus* biofilms with one or both of the biofilm degrading enzymes may enhance the susceptibility to antibiotics used in staphylococcal infections. We selected tobramycin for this investigation, as it is

active against many Gram-positive and Gram-negative pathogens and commonly used to reduce the bacterial burden of the lungs of cystic fibrosis patients co-infected with *S. aureus* and *P. aeruginosa*[18, 19].

Our study has demonstrated that treatment of pre-formed *S. aureus* biofilms with the biofilm-degrading enzymes DNase I or Dispersin B enhances the bactericidal activity of tobramycin, a model antibiotic used in this investigation. However, the antimicrobial efficacy of tobramycin was significantly reduced when pre-formed biofilms were treated with a combination of the two enzymes.

MATERIALS AND METHODS

Production and purification of recombinant dispersin B

Escherichia coli DH5α was transformed with the plasmid pDispersin applying standard procedures[20]. The plasmid consisted of the expression vector pASK-IBA33plus (IBA Lifesciences) containing a synthetic gene encoding dispersin B as described previously[21].

The *E. coli* strain containing pDispersin was grown overnight at 37ºC in LB broth (Oxoid) containing 300µg/mL ampicillin on an orbital shaker at 60rpm. Four 1-L Erlenmeyer culture flasks, each containing 400mL Terrific broth (Invitrogen) supplemented with 300µg/mL ampicillin were inoculated with 50µL of the *E. coli* culture and incubated overnight at 30ºC on a shaking incubator till OD_{600nm} reached ~2. Induction of recombinant dispersin B expression was achieved by addition of 200ng/mL anhydrotetracycline (Clontech). The flasks were placed on a shaking incubator at 37ºC for 2 hours. The bacteria were harvested by centrifugation (10,000g, 10 min, 4°C). The supernatant was discarded and the cell pellet resuspended in 16mL extraction buffer (20mM Tris-HCl, pH7.5, 500mM NaCl) containing 1mM PMSF, 2mg/mL lysozyme and 0.1% IGEPAL® (Sigma). The cell suspensions was sonicated 3 times for 10 sec at low amplitude, using a Diagenode Biorupter Plus device (10 sec pulse-off time), followed by incubation on ice for 30

min. DNase I was added to a final concentration of 5µg/mL, and RNase A to a final concentration of 10µg/mL, followed by incubated at room temperature for 30mins with gentle shaking.

After removal of the cell debris by centrifugation (15,000g, 30min, 4ºC), the supernatant was filtered through a 0.2µm filter. The filtered supernatant was passed through a His-Select Nickel Affinity Gel (Sigma-Aldrich) column with 8mL bed volume. The column was washed with 3 column volumes of extraction buffer before washing successively with extraction buffer containing 5mM and 20mM imidazole, respectively. Elution of dispersin B was achieved with 2 bed volumes of extraction buffer containing 100mM imidazole. Fractions (500µL) were collected and the OD_{280nm} of each fraction measured. Fractions containing the enzyme activity were pooled and dialysed overnight against 100mM phosphate buffer containing 200mM NaCl, pH 5.9. The dialysates was mixed with an equal volume of 20% glycerol in the same buffer before storing at -20ºC. The protein content was estimated by assuming that a 1mg/ml solution has an absorption value of 1.25 (http://web.expasy.org/protparam/). The dispersin B activity was confirmed using 4-nitrophenyl-N-acetyl-β-D-glucosaminide as a substrate as described by Kaplan et al. (2003).

Bacterial growth inhibition assay

The *S. aureus* isolate ATCC ® 29213™, a strong biofilm producer, was used in this investigation. The strain was grown overnight in Nutrient broth (NB, Oxoid) at 37ºC on an orbital shaker at 80rpm. The cells were washed twice by suspending pelleted cells in cold phosphate buffered saline (PBS pH 7.4, NaCl 137mmol/L, KCl 2.7mM/L, Na_2HPO_4 10mM, KH_2PO_4 1.8mM) and centrifugation at 15,000 rpm for 2 minutes.. The cell density was adjusted to an OD_{600nm} of 0.132 and the suspension placed on ice. A tobramycin dilution series ranging from 2µg/mL to 0.0625µg/mL was prepared in NB and dispensed in a 96-well microtiter plate (198µL/well). Two µL of the *S. aureus* suspension (0.132 OD_{600nm}) was added to each well, followed by

incubation at 37ºC and 80 rpm for 24 h. The OD_{600nm} was recorded and the sample values corrected by subtraction of the averaged blank values.

DNase I and dispersin B-mediated biofilm degradation

S. aureus biofilms were grown on 96-well plates as previously described[3]. After washing the biofilms twice with PBS, 100µL PBS containing either various concentration of DNase I (Sigma Aldrich, D4527, constituted from a freeze-dried vial at 50KU/mL and 140KU/mL) or dispersin B (720µg/mL, 500µg/mL, 100µg/mL, 50µg/mL, 10µg/mL) were added to each well. The plates were incubated on an orbital shaker for 2h at 37ºC and 50rpm (DNase I treatment) or 80rpm (dispersin B treatment). The remaining attached biofilms were washed once with PBS and stained for 5min with 0.2% crystal violet solution in PBS before washing twice with PBS. The crystal violet adsorbed to the residual biofilms was solubilized in 96% methanol and the OD_{630nm} recorded on an EnSpire Multimode plate reader (PerkinElmer). The OD_{630nm} value obtained for the negative control without biofilm was subtracted from the sample values.

Effect of combinations of biofilm degrading enzymes with tobramycin on bacterial cell viability

S. aureus ATCC ® 29213™, a strong biofilm forming strain, was grown overnight in NB supplemented with 1% glucose at 37ºC and then diluted 1:200 in the growth medium. The bacterial suspension was dispensed in a 96-well microtiter plate (200µL/well) and the plate incubated for 24 h at 37ºC on an orbital shaking platform at 60rpm, followed by incubation at 37ºC without shaking for an additional 24h. The biofilm formed on the bottom of the wells was washed twice with PBS. Solutions containing DNase I, dispersin B and tobramycin, alone or in different combinations, were prepared as specified in Table 1 and added to the bacterial suspensions in the wells (100µL/well) of the microtitre plate. The plate was incubated on an orbital

shaker for 2.5h at 37ºC and 60 rpm. The supernatant was aspirated from the treatment groups and remaining biofilms were scraped, suspended in 200µL of cold PBS and spread plated nutrient agar (Oxoid), followed by incubation at 37°C overnight. For validating the activity of dispersin B alone, a corresponding experiment was performed with biofilms produced by *Staphylococcus epidermidis.*

Table 1: Experimental design for assessment of the effects of singular use or combinations of biofilm-degrading enzymes with tobramycin *in vitro*.

Group	Composition	Concentration
1	Buffer control	PBS
2	Tobramycin	0.75µg/mL in PBS
3	DNase	140kU/mL in PBS
4	Dispersin B	0.72mg/mL Dispersin B
5	DNase + Dispersin B	140kU/mL DNase I, 0.72mg/mL, Dispersin B
6	DNase + tobramycin	0.75µg/mL tobramycin, 140kU/mL DNase I
7	Dispersin + tobramycin	0.75µg/mL tobramycin 0.72mg/mL Dispersin B
8	DNase, dispersin B and Tobramycin	0.75µg/mL tobramycin, 140kU/mL DNase, 0.72mg/mL dispersin B

Biofilm preparation for Scanning Electron Microscopy (SEM)

An overnight culture of *S. aureus* ATCC ® 29213™ in NB was pelleted in a 1.5-mL microcentrifuge tube (12,000rpm, 3 min). The cells were washed twice with 1mL ice-cold PBS and adjusted with NB to 10^8 CFU/mL. Aluminium stubs were placed upright in a 24-well plate containing NB with 1% glucose (2mL/well). To each well, 40µL of the bacterial suspension was added, and the plate incubated for 24h at 37ºC without shaking. The stubs were aseptically removed and gently washed with PBS. The stubs were placed into a new 24-well plate containing solutions of DNase I, dispersin B and tobramycin alone or in different combinations as specified in Table 1

(2mL/well). After incubation at 37ºC for 2.5h, the stubs were removed; their surface rinsed with PBS and allowed to dry at 37ºC for 30mins. To each stub surface 25µL 2.5% glutaraldehyde in PBS was added. After incubation for ≥3h at 4°C, the stubs were gently washed with water, dehydrated with 70%, 90% and 100% ethanol and placed into a desiccator for 3 days. The specimens were coated with 5nm platinum and analyzed using a Zeiss Neon 40ESB Crossbeam Electron Microscope.

RESULTS AND DISCUSSION

Biofilm-degrading activity of DNase I and dispersin B

The hypothesis underpinning our investigation was that combination of 2 biofilm-degrading enzymes will result in an improved dispersal of biofilms and antimicrobial efficacy of antibiotics in comparison with that obtained by the treatment of biofilms with the enzymes individually. The results obtained described in the text clearly did not validate this hypothesis.

Commercially available DNase I previously demonstrated to degrade eDNA in staphylococcal biofilms[21], and recombinant dispersin B known to cleave PNAG[19] were used in this investigation. Tobramycin, a broad-spectrum antibiotic, used in the treatment of patients with chronic ear infections[5], co-infected with *S. pneumoniae* and non-typeable *Haemophilus influenzae*, and cystic fibrosis patients frequently co-infected with *S. aureus* and *P. aeruginosa*[6] was used as a model antibiotic in this investigation.

In preliminary experiments, we confirmed that DNase I, applied at 140kU/mL, efficiently degraded pre-formed *S. aureus* biofilms grown on 96-well plates, leading to 65% reduction of staining with crystal violet compared to the untreated control ($p \leq 0.015$, data not shown). Also dispersin B, applied at 0.72mg/mL, partially degraded the *S. aureus* biofilm in this assay.

The specificity of the activity of the dispersin B preparation used in our study was confirmed by its ability to release the *p*-nitrophenolate moiety from the surrogate

substrate 4-nitrophenyl-N-acetyl-β-D-glucosaminide. The biofilm-degrading activity of this enzyme was also validated using biofilms formed by *S. epidermidis* used as a control in our investigation (data not shown).

Combined activity of biofilm-degrading enzymes with tobramycin

In a series of experiments, we studied the effect of the biofilm-degrading enzymes and the antibiotic tobramycin, alone and in combination, on the viability of *S. aureus* cells in the biofilm. In a standard broth microdilution assay, tobramycin partially suppressed the growth of *S. aureus* in the range of 0.5–1µg/mL (OD_{600nm}. <0.1). Thus, a tobramycin concentration of 0.75µg/ml was used in the subsequent experiments for the treatment of *S. aureus* biofilms. *S. aureus* biofilms grown on 96-well plates were incubated with the different enzyme and antibiotic solutions. Then the total number of viable *S. aureus* in each well, including the planktonic cells in the supernatant as well as the biofilm-associated cells, was determined. Cell scraper was used to ensure dislodging of the biofilm-embedded *S. aureus*. In all treatment groups the viability of the *S. aureus* cells was significantly affected, although to varying degrees (Table 2). Tobramycin alone resulted in 40-fold reduction, which is comparable to the effect of dispersin B alone (no significant difference). Significantly higher (p<0.004) efficacy was observed after treatment with DNase I alone resulting in 1285-fold reduction. Remarkably, the combination of DNase I with dispersin B reduced the cell viability less efficiently (p<0.005) than DNase alone indicating an inhibitory effect on the activity of dispersin B for DNase I. Most active were the combinations of tobramycin with DNase I (p<0.002) and tobramycin with dispersin B (p<0.004) resulting in 8780-fold and 7500-fold reduction, respectively. Unexpectedly, the triple combination of tobramycin, DNase I and dispersin B displayed comparably significantly less activity (129-fold reduction, p<0.034). The most probably explanation is the effect of one dispersal agent is disrupting the action of the other leading to a reduction in the efficacy of the antibiotic against *S. aureus*.

Treatment of *S. epidermidis* biofilms with dispersin B resulted in 12-fold reduction (1.2 $x10^{12}$ CFU/mL) compared to the untreated control (1.24 $x10^{13}$ CFU/mL) (data not shown in Table 2). Thus, dispersin B had a similar effect on the viability of *S. epidermidis* as on *S. aureus*.

Table 2: Total viable CFUs/mL after treatment of preformed *S. aureus* biofilms *in vitro* with biofilm-degrading enzymes, singularly or in combination with tobramycin

Treatment group	Active ingredients	Average CFU/mL	Log_{10} CFU/mL ± SE[a]	Fold reduction
1	Control	6.0 $x10^{11}$	11.76±0.09	N/A
2	Tobramycin	1.47 $x10^{10}$	10.14±0.12	40-fold
3	DNase I	4.67 $x10^{8}$	8.66±0.06	1285-fold
4	Dispersin B	9.37 $x10^{10}$	10.82±0.26	6.4-fold
5	DNase I + Dispersin B	1.87 $x10^{10}$	10.20±0.17	32-fold
6	DNase I + Tobramycin	8.0$x10^{7}$	7.90±0.00	8780-fold
7	Dispersin B + Tobramycin	6.83 $x10^{7}$	7.69±0.27	7500-fold
8	DNase I + Tobramycin + Dispersin B	4.67 $x10^{9}$	9.66±0.10	129-fold

[a]SE, standard error; N/A not applicable

Effect of biofilm-degrading enzymes and tobramycin on biofilm morphology

For studying the effect of the different treatments on the biofilm morphology, we allowed *S. aureus* biofilms to grow on aluminum stubs, which were then incubated

with the enzyme and antibiotic solutions before being processed for scanning electron microscopy. The untreated control biofilms displayed the characteristic features including sequential layering and the presence of well-developed channels that permit nutrients to flow through the biofilm (Fig. 1). The biofilm after tobramycin treatment was characterized by incomplete coverage of the substrate but still consisted of several cell layers. A largely reduced number of cells remaining attached to the substrate with more extended areas of complete dispersal was observed after all treatments containing DNase I and/or dispersin B. Apparently, the most efficient treatments for removal of the biofilm was the tobramycin-dispersin B followed by tobramycin-DNase I combination.

Figure 1 Scanning electron micrographs (magnification 6000-fold) showing *S. aureus* biofilms treated with buffer (Control) and after incubation with DNase I, dispersin B and tobramycin, alone and in combination, as indicated.

Figure 1(a) – Group 1: Control

Figure 1(b) – Group 2: Tobramycin

Figure 1(c) – Group 3: DNase I

Figure 1(d) – Group 4: Dispersin B

Figure 1(e) – Group 5: DNase I and dispersin B

Figure 1(f) – Group 6: DNase I and Tobramycin

Figure 1(g) – Group 7: Dispersin B and Tobramycin

Figure 1(h) – Group 8: Dispersin B, DNase I and Tobramycin

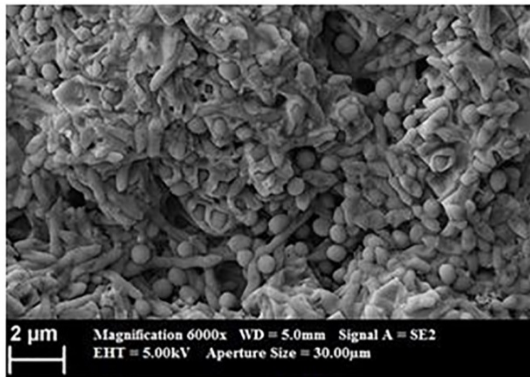

Fig 1a

Fig 1b

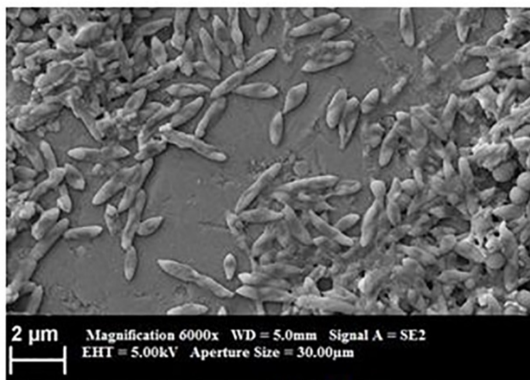

Fig 1c

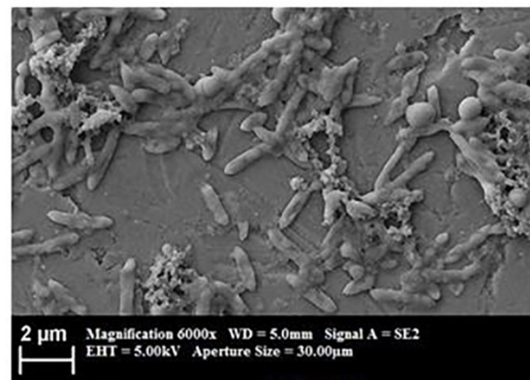

Fig 1d

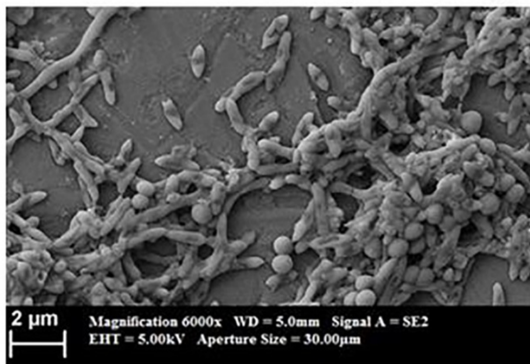

Fig 1e

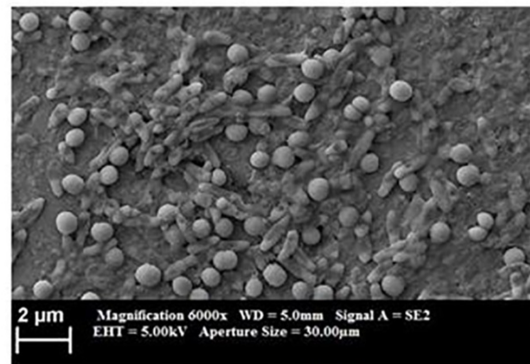

Fig 1f

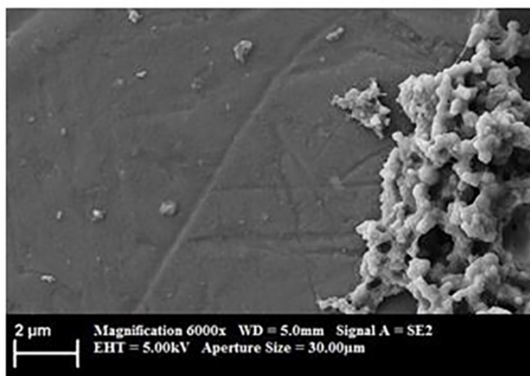

Fig 1g

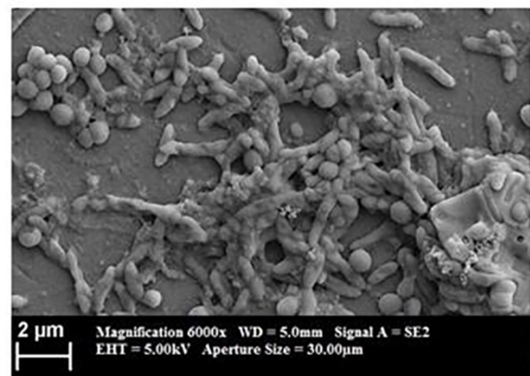

Fig 1h

Conclusions

Previous studies have demonstrated the efficacy of DNase I in degrading the biofilms of various Gram-positive and Gram-negative pathogens and its ability to enhance the bactericidal activity of several antibiotics[22]. However, no investigation thus far has studied the effect of a combination of biofilm degrading enzymes such as DNase and Dispersin B in combination with an antibiotic. Using *S. aureus* as a biofilm-forming pathogen, and tobramycin as a clinically relevant antibiotic, our study has demonstrated that use of multiple biofilm degrading enzymes in combination with each other does not necessarily result in a synergistic dispersal effect and may reduce the overall antimicrobial efficacy of the antibiotic Although the mechanism underlying the antagonistic effect of the two enzymes warrants further investigation, the outcome of this study suggests that combinations of different biofilm-degrading enzymes or compounds with antibiotics require careful assessment of their ability to enhance the efficacy of antibiotics *in vitro* before consideration for use in disinfecting or covalent coating of medical devices, or incorporation in pharmaceutical formulations targeted for use *in vivo*.

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ADDITIONAL INFORMATION

The author(s) declare no competing financial interests.

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Chapter 7

Waryah CB, Gogoi-Tiwari J, & Mukkur T (2015) Identification of a novel *Staphylococcus aureus* biofilm-associated antigen using proteomic analysis

Manuscript Submitted

Identification of a novel *Staphylococcus aureus* biofilm-associated antigen using proteomic analysis

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Key Words: *Staphylococcus aureus* biofilm antigen, immune proteomics

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Abstract

Staphylococcus aureus produces a wide variety of virulence factors, all influenced by bacteria's host environment. Over 50+ virulence factors have been identified which include capsular polysaccharide, poly-*N*-acetyl glucosamine (PNAG), exotoxins, enzymes and microbial surface components recognizing adhesive matrix molecules, (MSCRAMM). In this investigation, methicillin-sensitive *S. aureus* was grown using iron-replete versus iron deplete bacteriological media with a view to identify novel antigens associated with biofilm formation using preliminary proteomic analysis. One novel overexpressed band was isolated and purified. Fructose bisphosphate adolase Class 1 (FBA-1) was identified as an antigen associated with biofilm in S. aureus. FBA-1 showed little homology to FBA-1 of Homo sapiens based upon its protein sequence. FBA-1 is a highly conserved enzyme that is not only involved in essential metabolic pathways but also acts as a moonlighting protein. Because of the little homology of the protein sequence of *S. aureus* FBA-1 with *H. sapiens*, it was concluded that it could potentially be used as a target for the discovery of new antimicrobials and a potential vaccine candidate against infections caused by *S. aureus*.

Introduction

Staphylococcus aureus represents a major health concern as nosocomial and community acquired infections [1]. Treatment of *S. aureus* has become progressively challenging due to the emergence of antibiotics resistance due to biofilm formation [2, 3]. Virulence of *S. aureus* is attributed to the many factors including leucocidins, proteases, hemolysins, immune evasion molecules and molecules contributing to biofilm formation [4-6]. Bacterial infections are influenced by host environment which dictate expression of these virulence factors [7]. One important virulence attribute of most bacterial pathogens is the acquisition of iron for its survival in the host [8]. Iron redirects the central metabolism, including the iron and heme-dependent regulons of *S. aureus* [1, 9, 10], such that

metabolic blocks that hinder synthesis of virulence factors by *S. aureus* are avoided overcoming its vulnerability to host's immunological defences [11].

A different approach for proteomic analysis was undertaken in this investigation. *Staphylococcus aureus* was grown in iron-sequestering media using 2,2' Bipyridyl, an iron chelating compound to enhance the expression of iron and heme-dependent potential virulence genes [9]. One novel overproduced protein band was isolated and purified from 1D SDS-PAGE gel and identified to be fructose- 1,6-bisphosphate aldolase Class 1 by preliminary proteomic analysis.

Materials and Method

Optimizing bacterial growth in the presence of 2,2' Bipyridyl

A biofilm producing strain, HuAIRSF-S.aur23 (S23), selected for this investigation was grown overnight in 5mL of NB supplemented with 1% glucose (Glc) at 37°C. The culture was inoculated in NB (1% Glc) with or without 75µm of iron chelating agent 2,2' Bipyridyl (BP) and was grown for 24hr at 37°C on an orbital shaker. Growth of bacteria was determined at OD_{600nm}. To determine the effect of BP on biofilm, a 1 in 200 dilution of the culture was made in a 96 well plate and grown static for 24hr to develop biofilms iat 3 concentrations of BP. Supernatant was carefully aspirated and the biofilms were washed 2x with cold PBS. Biofilms were stained with 0.2% crystal violet for 5min, washed 2x with cold PBS and solubilized in methanol (96%). Measurements were taken at OD_{450nm}.

Preparation of whole cell lysates

The method for preparation was adapted from LaFrentz et al. [12]. To create biofilms, 100mL of NB (1% Glc) with or without BP was transferred to vented tissue culture flasks (Nunc). An inoculation with 20µL of the overnight culture was made and was incubated for 48hrs at 37°C.

Four types of lysates prepared from *S. aureus* grown in flasks were as follows:

1. Biofilm-embedded *S. aureus* grown in the presence of BP (BP+)
2. Free-floating (planktonic) *S. aureus* decanted from flasks grown BP+
3. Biofilm-embedded *S. aureus* grown in the absence of BP (BP-)
4. Free-floating (planktonic) *S. aureus* decanted from flasks grown BP-

Biofilm matured over 72hrs at 37°C without shaking and carefully handled to prevent disruption of biofilm. After 72hrs, supernatant was carefully poured off representing free-floating *S. aureus*. Cold PBS (100mL) was then added to each flask to wash biofilm bound bacteria. Cells were initially removed by manual agitation and followed by cell scrapers (ThermoFisher) to disperse any persistent biofilm. Biofilm-associated and free-floating cells were spun at 15,000g (20mins at 4°C) in Avanti® J-E Centrifuge (Beckman Coulter) and washed 3x in cold PBS.

Cells were resuspended in 50mL of PBS containing 0.5mg Lysozyme (Sigma) and 0.5mg Lysostaphin (Sigma) and incubated overnight at 37°C. Following incubations, cells were resuspended in 0.5mM PMSF (Sigma) 800µL/100mg wet cell. Cells were ribolyzed at 4°C in BIO101/Savant FastPrep FP120 at max speed for 45sec with acid-washed Glass beads (425-600µm, Sigma). Lysates were centrifuged at 15,000g (30mins at 4°C) and supernatant containing proteins was stored at -20°C until required. Protein content in the samples was estimated using the Micro BCA Assay (Thermo Fisher, Scoresby Vic) according to manufacturer's instructions.

Polyacrylamide Gel Electrophoresis

(a) SDS-PAGE

Discontinuous SDS-PAGE was performed to compare the lysates prepared using Novex® NuPAGE® SDS-PAGE gel system in the X-cell SureLock Mini cell system (Invitrogen, Life Technologies). A precast 10% NuPAGE® Bis-Tris gel (8x8cm, 1.0mm) was inserted into the mini cassette. The four whole cell lysates (20µg) were loaded and electrophoresed at 200V for 35min. BenchMark™ Protein Ladder (Novex®, Life Technologies) was run to estimate the molecular weight (mwt) of

proteins. Gels were stained in SimplyBlue™ SafeStain (Life Technologies) and visualized on the ChemiDoc™ MP System (Biorad). For the isolation of preparative amounts of the unique band, SDS-PAGE using 20% polyacrylamide was used.

(b) Western Blotting

Following SDS-PAGE, proteins were transferred onto nitrocellulose membrane (0.45µm, Biorad) using XCell SureLock™ Transfer System (Life Technologies). Following assembly of the transfer sandwich cassette, the system was run for 1hr at 30V constant. The MagicMark™ XP Western Protein Standard (Life Technologies) was used for mwt estimation of proteins.

Unless otherwise stated, all washing, blocking and incubation steps were performed on an orbital shaker at 120rpm. The blot was removed, washed 3 times in TBST (5min each wash) and blocked at RT for 1hr in 3% BSA in 1x TBST. The blot was incubated overnight with rabbit anti-*S. aureus* primary antibody (1:2,500, Abnova) at 4ºC. The blot was rinsed 3 times (5 mins each wash) with TBST and incubated with secondary anti-rabbit antibody for 1hr at RT. The blot was rinsed 3 times (5 mins each wash) with TBST and Immun-Star™ Goat Anti-Rabbit AP Chemiluminescence Kit (Biorad) was applied according to manufacturer's recommendation as a substrate. The ChemiDoc™ MP System (Bio-Rad) imager was used to capture the signals of band.

Protein Identification by Mass Spectrometry

A protein of interest (30 - 40 kDa) identified to be unique in virulence was aseptically sliced out of a Coomassie stained gel. The section was stored at -20ºC until sequencing by Mass Spectrometry was performed by Proteomics International. The section was trypsin digested [13] and extracted according to standard procedures before analysis on 5800 Proteomic Analyzer (AB Sciex). Identification was performed using a software matching system (Mascot – Matrix Science) with a Ludwig NR Database.

Bioinformatics analysis

Following Proteomic analysis, the protein sequence was compared to other bacteria. Sequences, obtained from NCBI databases from 8 bacteria were compared for level of similarity with human FBA-1. The bacteria used included Gram-positive *S. aureus*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Staphylococcus epidermidis* and Gram-negative *Pseudomonas aeruginosa*, *Neisseria meningitidis*, *Campylobacter jejuni* and *Escherichia coli*. A multiple sequence alignment was created using ClustalW2.1 program and a protein identity similarity index created.

Results

Standardisation of cultural conditions

The effect of 3 concentrations of BP on the growth of *S. aureus* was determined for optimal growth and biofilm formation [9, 14], ensuring the availability of iron of iron-sequestering protein, capable of capturing iron from the host. Concentration of BP selected for this experiment was 75µM to obtain adequate growth and biofilm formation (Table 1).

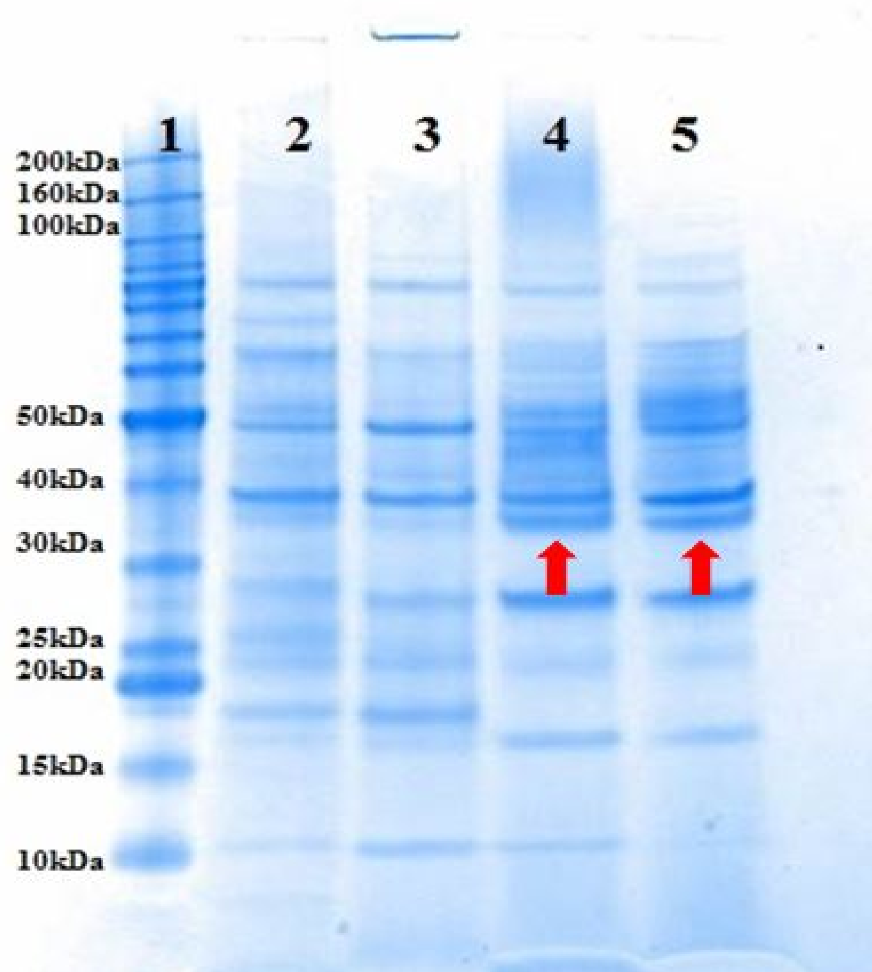
Table 1: Determination of bacterial growth conditions for optimal production of biofilms of *S. aureus* in the presence of BP at OD_{600nm}

Parameter	50µM	75µM	100µM
Bacterial cell density at 600nm ± SEM	0.486 ± 0.052	0.518 ± 0.029	0.418 ± 0.006
Percent reduction	4.8%	11%	18.2%
Absorbance of lysed biofilm measured at 450nm	0.074 ± 0.000	0.073 ± 0.001	0.059 ± 0.001

Purification of biofilm-associated band

Upon 1-D SDS PAGE, a unique band between 30-40kDa, which was under-expressed supernatant and biofilm (BP−) cells of *S. aureus*, was overexpressed in the supernatant and biofilm (BP+) cells (Fig 1).

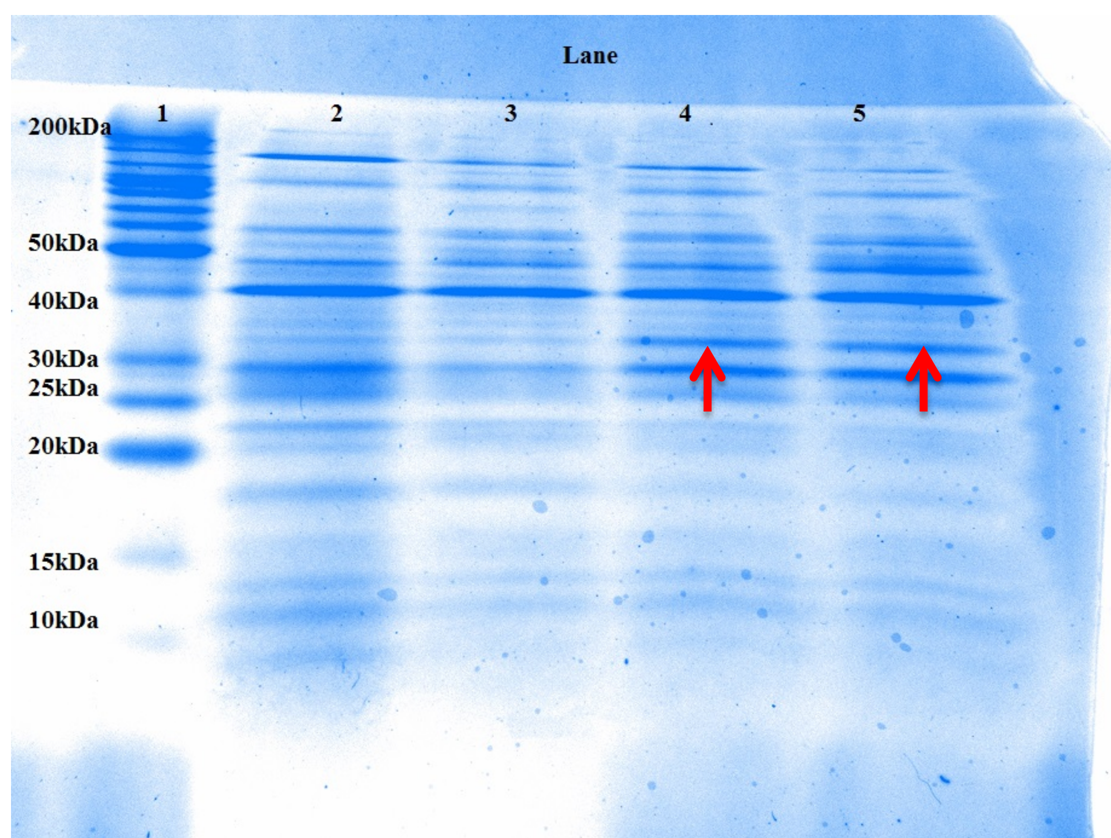
Fig 1: SDS-PAGE of *S.aureus* grown in BP+ or BP- media



Lane 1 - BenchMark Protein ladder with approximate mwt. Lane 2 – Protein lysate of supernatant BP- *S.aureus* cells, Lane 3 – Protein lysate of biofilm cells BP-, Lane – 4 - Protein lysate of supernatant cells BP+ , Lane 5 – Protein lysate of biofilm cells BP+. Differential overexpressed protein can be observed in lanes 3 and 4 between molecular mass of 30 and 40kDa. Several additional bands can be observed between the 4 lysates between 15 and 20kDa in lanes 2 and 4. However, the overexpression of the differential band in lanes 4 and 5 was more prominent.

Using the PROTEAN® II XL Cell system (Biorad) a hand-cast 20% gel (Acrylamide/Bis 29:1, Sigma) was prepared. The gel was run at were run overnight at 6-8mA constant using a PowerPac™ power supply (Biorad) and 10ºC cooling system with water circulating pump . The higher percentage of polyacrylamide gel allowed for better separation of this relatively lower mwt protein band (Fig 2).

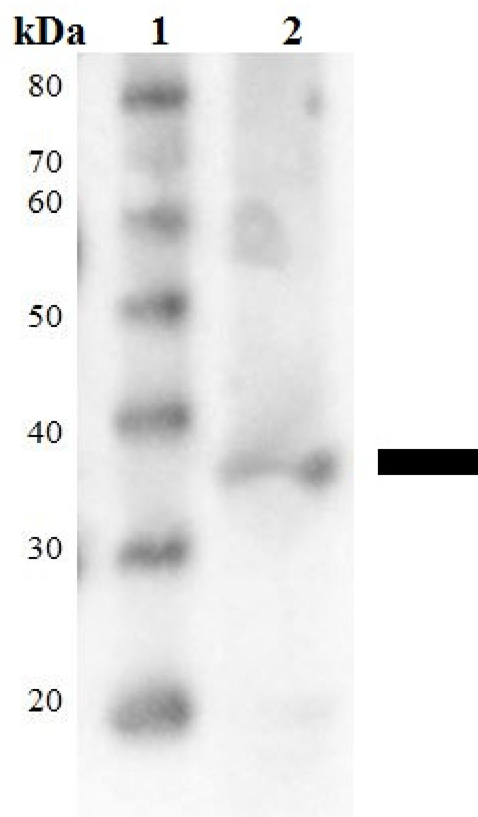
Fig 2: Discontinuous SDS-PAGE (20%) for isolation of unique protein band



Lane 1: BenchMark Protein ladder with approximate mwt. Lane 2 – Protein lysate of supernatant BP- *S.aureus* cells, Lane 3 – Protein lysate of biofilm cells BP-, Lane – 4 - Protein lysate of supernatant cells DP+ , Lane 5 – Protein lysate of biofilm cells BP+. The same overexpressed differential protein can be observed in lanes 3 and 4 between 30 and 40kDa.

The gel band stained with Coomassie blue was aseptically sliced out with a sterile scalpel submitted for identification by Mass Spectrometry [13] . Protein was checked for purity by Western blotting using anti-*S. aureus* antibody (Fig 3) when a single relatively diffuse band of ~33 kDa was observed.

Fig 3: Western blot of purified protein



Lane 1 contains the MagicMark™; Lane 2 contains purified FBA; Lane 3 is an artificial representation of the location of the FBA.

Mass Spectrometry predicted the protein band to be Fructose-bisphosphate adolase Class 1, FBA-1, approximately 32.8kDa with a score of 593. Score greater of each peptide sequence match was enough to confirm identity. Seven peptides were matched for FBA-1 (Fig 4) which identities higher than the cut off and had a significant threshold of $p<0.05$. Interestingly, the band was identified after covering only 27% of *S. aureus* genome sequence (Tax_Id=948561 *Staphylococcus aureus* O11).

Fig 4: Protein identification of FBA-1

Protein sequence coverage: 27%

Matched peptides shown in ***bold red***.

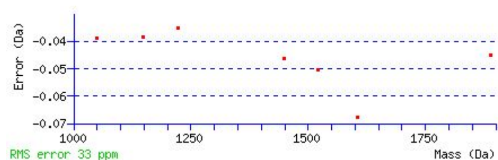
```
  1 MNKEQLEKMK NGKGFIAALD QSGGSTPKAL KEYGVNEDQY SNEDEMFQLV
 51 HDMRTRVVTS PSFSPDKILG AILFEQTMDR EVEGKYTADY LADKGVVPFL
101 KVDKGLAEEQ NGVQLMKPID NLDSLLDRAN ERHIFGTKMR SNILELNEQG
151 IKDVVEQQFK VAKQIIAKGL VPIIEPEVNI NAKDKADIEK VLKAELKKGL
201 DSLNADQLVM LKLTIPTEAN LYKDLAEHPN VVRIVVLSGG YSREKANELL
251 KDNAELIASF SRALASDLRA GQSKEEFDKA LGDAVESIYD ASVNKN
```

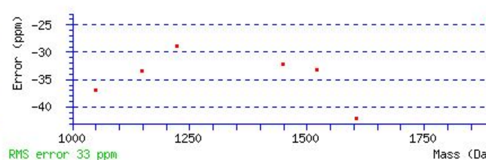
Unformatted sequence string: 296 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
37	14 – 28	1448.6915	1447.6842	1447.7307	-0.0465	0	99	3.1e-06	1		K.GFIAALDQSGGSTPK.A
40	68 – 80	1522.7428	1521.7355	1521.7861	-0.0506	0	25	73	3		K.ILGAILFEQTMDR.E + Oxidation (M)
45	169 – 183	1605.8534	1604.8461	1604.9137	-0.0676	0	60	0.023	1		K.GLVPIIEPEVNINAK.D
21	224 – 233	1149.5627	1148.5554	1148.5938	-0.0384	0	82	0.00019	1	U	K.DLAEHPNVVR.I
15	234 – 243	1050.5555	1049.5482	1049.5869	-0.0387	0	84	0.00012	1	U	R.IVVLSGGYSR.E
50	246 – 262	1890.9467	1889.9394	1889.9846	-0.0452	1	142	1.2e-10	1	U	K.ANELLKDNAELIASFSR.A
29	252 – 262	1222.5709	1221.5636	1221.5989	-0.0353	0	99	3.4e-06	1	U	K.DNAELIASFSR.A





Multiple Sequence Alignment

The protein sequence of *S. aureus* FBA-1 was aligned with other species using the ClustalW2.1 sequence alignment program to determine similarities (Fig. 5).

Fig. 5: Sequence alignment using the ClustalW2.1 program

```
CLUSTAL 2.1 multiple sequence alignment


S.pneumoniae        -------------------------MAIVSAEKFVQAARDNGYAVGGFNTNNLEWTQAIL 35
S.agalactiae        -------------------------MAIVSAEKFVQAARDNGYAVGGFNTNNLEWTQAIL 35
S.epidermidis       -------------------------MPLVSMKEMLIDAKENGYAVGQYNLNNLEFTQAIL 35
P.aeruginosa        -------------------------MALISMRQMLDHAAEFGYGVPAFNVNNLEQMRAIM 35
N.meningitidis      MSRLWFFAAKNIIIRLIYLLPKETQMALVSMRQLLDHAAENSYGLPAFNVNNLEQMRAIM 60
C.jejuni            --------------MGVLDIVKAGVISGDELNKIYDYAKAEGFAIPAVNVVGTDSINAVL 46
E.coli              --------------MTDIAQLLGKDADNLLQHRCMTIPSDQLYLPGHDYVDRVMIDNNRP 46
S.aureus            -------------MNKEQLEKMKNGKGFIAALDQSGGSTPKALKEYGVNEDQYSNEDEMF 47
H.sapiens           -------------MPHSYPALSAEQKKELSDIALRIVAPGKGILAADESVGSMAKRLSQI 47
                                                         .                      

S.pneumoniae        -----RAAEAKKAPVLIQTSMGAAKYMGGYKVARNLIANLVESMG--------ITVPVAI 82
S.agalactiae        -----RAAEAKKAPVLIQTSMGAAKYMGGYKLCKQLIETLVESMG--------ITVPVAI 82
S.epidermidis       -----EASQEENAPVILGVSEGAARYMSGFYTVVKMVEGLMHDLN--------ITIPVAI 82
P.aeruginosa        -----EAADKTDSPVIVQASAGARKYAG-APFLRHLILAAIEEF---------PHIPVVM 80
N.meningitidis      -----EAADQVNAPVIVQASAGARKYAG-APFLRHLILAAVEEF---------PHIPVVM 105
C.jejuni            -----EAAKKVNSPVIIQFSNGGAKFYAGKNCPNGEVLGAISGAKHVHLLAKAYGVPVIL 101
E.coli              -----PAVLRNMQTLYNTGRLAGTGYLSILPVDQGVEHSAGASFAAN-PLYFDPKNIVEL 100
S.aureus            -----QLVHDMRTRVVTSPSFSPDKILGAILFEQTMDREVEGKYT------------ADY 90
H.sapiens           GVENTEENRRLYRQVLFSADDRVKKCIGGVIFFHETLYQKDDNGVPFVRTIQDKGIVVGI 107
                                  :            .                             .  

S.agalactiae        HLDHG--------------HYDDALECIEVGYTS---IMFDGSHLP-------VEENLEK 118
S.epidermidis       HLDHGS-------------SFEKCKEAIDAGFTS---VMIDASHSP-------FEENVEI 119
P.aeruginosa        HQDHGT-------------SPDVCQRSIQLGFSS---VMMDGSLREDGKTPADYDYNVRV 124
N.meningitidis      HQDHGA-------------SPDVCQRSIQLGFSS---VMMDGSLMEDGKTPSSYEYNVNA 149
C.jejuni            HTDHAARKLLPWIDGLIEANAQYKKTHGQALFSS---HMLDLSEES-------LEENLST 151
E.coli              AIEAGCN---------CVASTYGVLASVSRRYAHRIPFLVKLNHNETLSYPNTYDQTLYA 151
S.aureus            LADKG----------------VVPFLKVDKGLAE---EQNGVQLMKP-------IDNLDS 124
H.sapiens           KVDKG----------------VVPLAGTDGETTTQGLDGLSERCAQYKKDGADFAKWRCV 151
                      : .                       .   :                           

S.pneumoniae        AKEVVEKAHAKGISVEAEVGTIG-------GEEDGIIGKGEL-------APIEDAKAMVE 164
S.agalactiae        AREVVAKAHAKGISVEAEVGTIG-------GEEDGIVGKGEL-------APIEDAKAMVE 164
S.epidermidis       TSKVVEYAHDRGVSVEAELGTVG-------GQEDDVVADGVIYA-----DPKECQELVEK 167
P.aeruginosa        TQQTVAFAHACGVSVEGELGCLGSLETGMAGEEDGVGAEGVLDHSQLLTDPEEAADFVKK 184
N.meningitidis      TRTVVNFSHACGVSVEGEIGVLGNLETGEAGEEDGVGAVGKLSHDQMLTSVEDAVRFVKD 209
C.jejuni            CEVYLQKLDALGVALEIELGCTG-------GEEDGVDNTGIDNS-KLYTQPEDVALAYER 203
E.coli              SVEQAFNMGAVAVGATIYFGSEE--------SRRQIEEISAAFERAHELGMVTVLWAYLR 203
S.aureus            LLDRANERHIFGTKMRSNILELN---------EQGIKDVVEQ--------QFKVAKQIIA 167
H.sapiens           LKISERTPSALAILENANVLARYAS----ICQQNGIVPIVEPEILPDGDHDLKRCQYVTE 207
                               .      .             .  :                        

S.pneumoniae        TG----IDFLAAGIGNIHGPY---------PVNWEGLDLDHLQKLTEALPGFPIVLHGGS 211
S.agalactiae        TG----IDFLAAGIGNIHGPY---------PANWEGLDLDHLKKLTEAVPGFPIVLHGGS 211
S.epidermidis       TG----IDTLAPALGSVHGPY---------KGEPK-LGFKEMEEIGAST-GLPLVLHGGT 212
P.aeruginosa        TK----VDALAIAIGTSHGAYK-----FTKPPTGDTLSIQRIKEIHARIPDTHLVMHGSS 235
N.meningitidis      TG----VDALAIAVGTSHGAYK-----FTRPPTGDVLRIDRIKEIHQALPNTHIVMHGSS 260
C.jejuni            LGKISDKFSIAASFGNVHGVYKPGNVSLQPEILKNSQKFVKDKFALNSDKPINFVFHGGS 263
E.coli              NSAFKKDGVDYHVSADLTGQAN-----HLAATIGADIVKQKMAENNGGYKAINYGYTDDR 258
S.aureus            KG----LVPIIEPEVNINAKDK---------ADIEKVLKAELKKGLDSLNADQLVMLKLT 214
H.sapiens           KVLAAVYKALSDHHVYLEGTLLKPN--MVTPGHACPIKYTPEEIAMATVTALRRTVPPAV 265
```

```
S.pneumoniae        ---------------------GIPDEQIQAAIKLGVAKVNVNTECQIAFANATRKFARD- 249
S.agalactiae        ---------------------GIPDDQIQEAIKLGVAKVNVNTECQLAFCQATRDYAVE- 249
S.epidermidis       ---------------------GIPTKDIQKAIPYGTAKINVNTENQIASAKAVR------ 245
P.aeruginosa        SVPQDWLAIINEYGGEIKETYGVPVEEIVEGIKYGVRKVNIDTDLRLASTGAIR------ 289
N.meningitidis      SVPQEWLKVINEYGGNIGETYGVPVEEIVEGIKHGVRKVNIDTDLRLASTGAVR------ 314
C.jejuni            ---------------------GSELKDIKNAVSYGVIKMNIDTDTQWAFWDGVREYELKN 302
E.coli              ------------------VYSKLTSENPIDLVRYQLANCYMGRAGLINSGGAAG------ 294
S.aureus            --------------------IPTEANLYKDLAEHPNVVRIVVLSGGYSREKANELLKDN- 253
H.sapiens           PG------------VTFLSGGQSEEEASFNLNAINRCPLPRPWALTFSYGRALQASAVN- 312
                                             .                         .

S.pneumoniae        --------YEANEAEYDKKKLFDPRK---FLADGVKA-IQASVEERIDVFGSEGKA---- 293
S.agalactiae        --------FNANEAEYMKKKLFDPRK---FLKPGFDA-ITEAVEERIDVFGSANKA---- 293
S.epidermidis       ------------EVLNNDKDVYDPRK---YLGPAREA-IKETVKGKIREFGTSNRAK--- 286
P.aeruginosa        ------------RFLAQNPSEFDPRK---YFSKTVEA-MRDICIARYEAFGTAGNASKIK 333
N.meningitidis      ------------RYLAENPSDFDPRK---YLSKTIEA-MKQICLDRYLAFGCEGQAGKIK 358
C.jejuni            RAYLQGQIGNPEGDDKPNKKYYDPRV---WLRSGEES-MIKRLEIAFEDLNCINKN---- 354
E.coli              --------GETDLSDAVRTAVINKRAGGMGLILGRKA-FKKSMADGVKLINAVQDVYLDS 345
S.aureus            ----------AELIASFSRALASDLR----AGQSKEE-FDKALGDAVESIYDASVNKN-- 296
H.sapiens           -----AWRGQRDNAGAATEEFIKRAEVNGLAAQGKYEGSGEDGGAAAQSLYIANHAY--- 364
                                          .                           :

S.pneumoniae        ---------------------
S.agalactiae        ---------------------
S.epidermidis       ---------------------
P.aeruginosa        PISLEGMFQRYARGELDPKVN 354
N.meningitidis      PVSLEKMANRYAKGELNQIVK 379
C.jejuni            ---------------------
E.coli              KITIA---------------- 350
S.aureus            ---------------------
H.sapiens           ---------------------
```

The percent identity matrix was generated using the multiple sequence alignment showing very little similarity between *S. aureus* FBA-1 and FBA-1 from the remaining species (Table 2).

Table 2: Percent Identity Matrix – created by ClustalW2.1

	S. pneumoniae	*S. agalactiae*	*S. epidermidis*	*P. aeruginosa*	*N. meningitidis*	*C. jejuni*	*E.coli*	*S. aureus*	*H. sapiens*
S. pneumoniae	100.00	87.71	46.45	38.30	40.07	27.99	10.45	11.47	11.34
S. agalactiae	87.71	100.00	47.16	39.01	40.07	29.35	8.36	12.54	12.37
S. epidermidis	46.45	47.16	100.00	39.79	38.38	29.12	8.77	8.86	10.25
P. aeruginosa	38.30	39.01	39.79	100.00	77.97	30.03	10.26	10.18	10.93
N. meningitidis	40.07	40.07	38.38	77.97	100.00	28.62	9.91	11.15	9.29
C. jejuni	27.99	29.35	29.12	30.03	28.62	100.00	10.80	10.31	8.18
E.coli	10.45	8.36	8.77	10.26	9.91	10.80	100.00	8.28	8.66
S. aureus	11.47	12.54	8.86	10.18	11.15	10.31	8.28	100.00	13.56
H. sapiens	11.34	12.37	10.25	10.93	9.29	8.18	8.66	13.56	100.00

Discussion

Fructose-bisphosphate aldolase, FBA-1 or aldolase, is a highly conserved enzyme that is involved in glycolysis and gluconeogenesis [15, 16]. Aldolase performs an aldol reaction by reversibly catalysing dihydroxyacetone phosphate with glyceraldehyde 3-phosphate to form fructose 1,6-bisphosphate [17]. Interestingly, enzymes also play a role in virulence as exemplified by interaction of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme that also interacts with aldolase in glycolysis found performing non-glycolytic activities on the surface of Gram-positive bacteria, including *S. aureus* [18, 19]. In *S. aureus*, a study identified GAPDH as a cell wall protein that binds to iron-binding transferrin, that is known to enhance virulence of pathogens [20, 21]. The function of aldolase has also been explored further as an increasing number of glycolytic pathway enzymes being classified as moonlighting enzymes such as GAPDH [22].

Aldolases are cytoplasmic enzymes that have been found localized on bacterial membrane for interaction with host molecules [16]. Tunio et al., [16] further characterized the ability of aldolase in adhesion to host human cells where aldolase deficient *Neisseria meningitidis* showed a significant reduction in adherence to two different cell types; human brain microvascular endothelial cells and human larynx carcinoma cells. Another study demonstrated that aldolase from *Streptococcus pneumoniae* had no human orthologues [23]. A result confirmed in this study using a multiple sequence alignment including *S. aureus* which shows 13.56% identity with human aldolase. Blau et al. [24] demonstrated recombinant aldolase and anti-aldolase antibodies were able to inhibit encapsulated and non-capsulated *S. pneumonia* from binding to lung carcinoma epithelial cells. This highlights the potential of aldolase in biofilm formation, a significant virulence factor in establishment and persistence of infections caused by *S. aureus* [3, 25, 26].

Ling et al. [23] demonstrated that immunization of mice with aldolase conferred protection when challenged with pneumococci with a survival rate, at 21 days post-challenge, of 36% [23].

In this investigation, FBA-1 has, for the first time, been identified as the overexpressed virulence protein associated with biofilm formation in *S. aureus*. The molecular mass of this enzyme is approximately 33 kDa, which is in compliance with that reported previously reported [27]. A previous investigation reported the presence of FBA Class 2 in planktonic cultures of *S. aureus* [28] but not in biofilms. Because of low homology of FBA Class 1 with the human aldolase, further investigations of its virulence and protective potential against infections caused by *S. aureus* are justifiably warranted.

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Chapter 8

Waryah CB, Gogoi-Tiwari J, Sunagar R, Isloor S, Hedge N, & Mukkur T (2015) Comparative immunogenicity and protective potential fructose-bisphosphate aldolase Class 1 versus manganese transport protein C of *Staphylococcus aureus* in mice

Manuscript Submitted

Comparative immunogenicity and protective potential fructose-bisphosphate aldolase Class 1 versus manganese transport protein C of *Staphylococcus aureus* in mice

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Key Words: Fructose-bisphosphate aldolase, infection, mouse model, immune response

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Abstract

Fructose-bisphosphate aldolase class 1 (FBA-1) a moonlighting protein, was identified as the novel antigen that was overproduced by *S. aureus* grown an iron-deplete environment. FBA-1 was purified from the biofilm of *S. aureus* and tested for its passive protective potential versus that of the manganese transport protein C (MntC), an accredited biofilm associated antigen, using an acute murine bacteraemia model. The immunogenicity of the MntC was found to superior to that of FBA-1. The major antibody isotype induced by FBA-1 was IgM whereas immunisation with MntC resulted in production of significant levels of IgG1 and IgG2a isotypes. Antibodies against FBA-1 were able to significantly lower the bacterial load in spleen but not so in blood and liver. It is concluded that MntC is a superior potential vaccine candidate for than FBA-1 for the prevention of staphylococcal infection in mice.

Introduction

Staphylococcus aureus remains a challenging infection to treatment due to the pathogen's ability to produce a wide variety of virulence factors. [1, 2] The regulation and production of virulence factors by *S. aureus* is largely dependent on host environment [3] and one such factor is the ability to acquire iron from the host for bacterial survival. [4] Iron has been shown to be involved in the regulation of the expression of proteins, many of which are involved in central metabolic pathways in *S. aureus.* [5]

One such protein is fructose-bisphosphate aldolase class 1, hereafter referred to as FBA-1. Under the stress of an iron sequestering compound, 2,2' bipyridyl, FBA-1 was identified as a 33kDa biofilm-associated protein expressed by *S. aureus*. FBA-1 is conserved cytoplasmic enzyme that catalyses the reversible reaction of splitting the aldol, fructose 1, 6-bisphosphate into triose phosphates, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. [6-8] Despite the lack of secretion signals, FBA-1 is expressed on the surface of many bacterial pathogens participating in the adhesion

and biofilm formation [7, 10, 11] acting as a moonlighting protein. [6] A previous study reported that FBA-1 of *S. aureus* was only expressed under planktonic or free-floating conditions.[7] This is in contrast to our finding that FBA-1 was produced in both the biofilm as well as planktonic cells of *S. aureus* (Unpublished).

Manganese transport protein C (MntC) is a conserved manganese binding surface lipoprotein that has been shown to be involved in biofilm formation and *in vivo* survival in the host. [8]

In this investigation, *S. aureus* purified FBA-1 and recombinant MntC were evaluated for their immunogenicity and protective potential using an acute murine bacteraemia model. Antibodies raised were then tested for their passive protective potential against acute bacteraemia caused by *S. aureus* using a mouse model. Results were compared to immune response raised by *S. aureus* MntC.

Materials and Methods

Isolation of Fructose-bisphosphate aldolase Class I

Protein lysates were prepared as previously described using strain HuAISRF-S.aur23 or S23 (Waryah, unpublished). Briefly S23 was grown in vented tissue culture flasks in 100mL NB (1% glucose) with the iron chelating agent 2,2' Bipyridyl or BP (75µm). Biofilm was allowed to develop for 72hr at 37˚C after the supernatant was carefully aspirated. Biofilm bound cells were initially removed by manual agitation by washing with cold PBS then by using cell scrapers (Thermofisher) to scrape any persistent biofilms. Cells were spun down at 15,000g for 20mins at 4°C followed by overnight incubation in 50mL PBS with 0.5mg Lysozyme (Sigma) and 0.5mg Lysostaphin (Sigma). Following incubation the cells were suspended in 800µL of 0.5mM PNSF (Sigma) per 100mg wet cell weight. For final lysis, cells were ribolyzed at 4°C in BIO101/Savant FastPrep FP120 at max speed for 45sec using acid-washed Glass beads (425-600µm, Sigma). Finally proteins were collected from the supernatant after centrifugation at 15,000g for 30mins at 4°C.

The lysates were subjected to discontinuous SDS-PAGE electrophoresis 20% polyacrylamide gel in the PROTEAN® II XL Cell system (Bio-Rad) for isolation of the ~33kDa band identified as fructose 1,6-bisphosphosphate aldolase class 1 (FBA-1) (Unpublished). One lane contained pre-stained ladder and 14 lanes contained protein lysate. PROTEAN® II XL Cell system (Bio-Rad) was cooled at 10ºC constant using a water circulation pump and two SDS-PAGE gels run overnight at 6-8mA constant. After completion of electrophoresis, individual lanes from each gel were sliced out and fixed in 40% methanol containing 10% acetic acid for 15min at RT on an orbital shaker at 80rpm. The gel slices were stained with Coomassie R-250 (0.02% in 30% methanol, 10% acetic acid) at RT for 30min at RT on an orbital shaker at 80rpm. The gel slices were destained in 8% acetic acid until a clear background was obtained. Bands corresponding to ~33kDa were sliced out with a clean scalpel, minced into smaller gel fragments and suspended in 400µL elution buffer (0.25 M Tris-HCl buffer, pH 6.8; 0.1% (w/v) SDS). The protein sections in elution buffer were transferred into 100k molecular weight cut off (MWCO) Nanosep® Centrifugal Devices (Pall Corporation) for protein concentration and separation from polyacrylamide gel following centrifugation at 14,000g for 15mins at 4ºC. A second elution was carried out using the same protocol to ensure a higher protein recovery. Protein quantification was performed using the Micro BCA Assay as per manufacturer's instructions (ThermoFisher).

Removal of SDS from extract

Prior to animal trials, ProteoSpin™ Detergent Clean-Up Micro Kit (Norgen Biotek Corp, Millennium Science Pty Ltd) was implemented to remove SDS from the eluted protein as per manufacturer's instructions.

Polymerase Chain Reaction of MntC gene

DNA extraction of S23 was performed using the MO-BIO commercial extraction kit and stored at -20°C. DNA extract was thawed on ice prior to genotyping using PCR.

Primers for the detection of *MntC* were designed in this study using the online Primer3plus primer design tool with *S. aureus* MntC gene sequence deposited on Genbank, NCBI. Primer mntCF 5'-CATGGCACGTTGTTCTTTTG-3' and Primer mntCR 5'-TGGTGGAGACAACGTCGATA-3' were used with the following conditions: 95°C for 10min (initial denaturation), then 32 cycles of 95°C for 30sec (denaturation), 49°C for 30sec (annealing) and 72°C for 1min (extension) and 72°C for 7min (final extension). PCR products were run on a 1.5% agarose gel in 1x Sodium Borate buffer (10 mM NaOH, pH 8.5 with H_3BO_3). Gels were stained with 0.8µL/100mL Midori Green DNA Stain (Nippon Genetics) and visualized using a UV transilluminator.

Purification of MntC

The MntC was cloned and expressed into a pRSetA bacterial expression vector with the incorporation of a His-tag. Following cloning, plasmid was transformed into *E.coli* DH5α cells and plated to screen for plasmid containing colonies using ampicillin as a selction marker. The plasmid was isolated and expressed in *E.coli* BL21 cells with the induction of the MntC protein with IPTG. Cells were harvested and denatured with 8M urea. Cell lysates were passed through a Ni-NTA column for purification of the His-tagged MntC. Purified MntC was confirmed by SDS-PAGE and Western blotting to be 38kDa protein.

Production of antisera and experimental design

All animals used in this investigation were approved by the Curtin University Animal Ethics Committee (AEC approval number 042-2014). Three groups of 5-6

week-old Balb/c mice were used in this investigation. Mice in groups 1 and 2 were immunised with FBA-1 and MntC respectively. The third group comprising the control group was sham-vaccinated with sterile PBS.

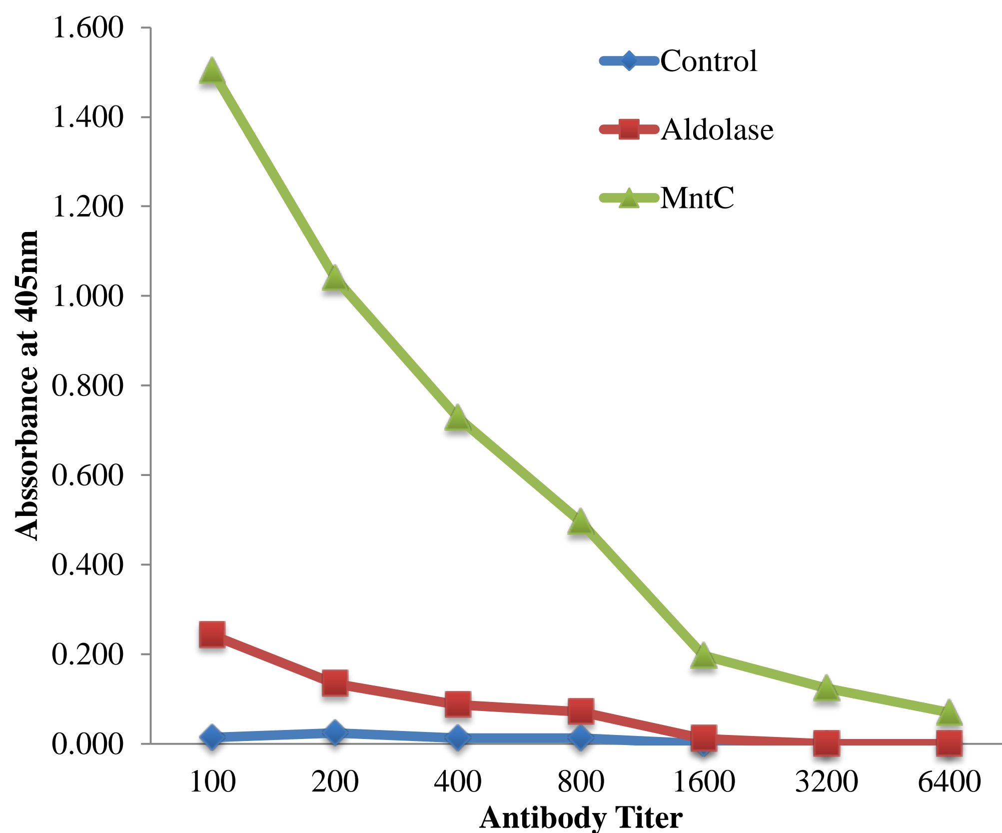
The immunisation regime was the same for all the groups. While the control group of mice were sham-immunised with sterile PBS, those in groups 1 and 2 were immunized with two doses, 2µg and 4µg, of FAB-1 and MntC respectively. The 3rd and 4th doses of 6 µg and 10 µg of each antigen were mixed with equal amounts of Alumjet (Thermo-Fisher) before administration via the subcutaneous route. The immunisation or sham-immunisation regime involved delivery of the different doses on day 0, 7, 14 and 18. All mice were euthanized on the 23rd day and blood samples were collected via cardiac puncture. The method used for semi-quantification of the different antibody isotypes (IgG1, IgG2a, IgM and IgA) levels was indirect ELISA as described elsewhere. [9]

The protective potential of FAB-1 and MntC was assessed by passive immunisation of mice with 0.5mL of antigen-specific antisera or PBS representing the controls via the intraperitoneal route. The vaccinated or sham-vaccinated mice were allowed to rest for 2 days, followed by bacterial challenge with $1x10^7$ CFU/mL HuAIRSF-S.aur23 (S23). Four hours post-challenge, mice were euthanized for collection of blood in a microfuge tube containing an anticoagulant (4% solution of sodium citrate in sterile dDW, pH 7.0), spleen and liver to determine bacterial burden as described elsewhere (Anderson et al.,2012). Briefly, blood was serially diluted and plated to enumerate bacterial load in CFU/mL. Liver and spleen were weighed and homogenized prior to serial dilution to enumerate the bacterial load (CFU/mL) per gram of tissues. Statistical analysis was performed using paired Student t-test and p-value≤0.05 was considered statistically significant.

Results

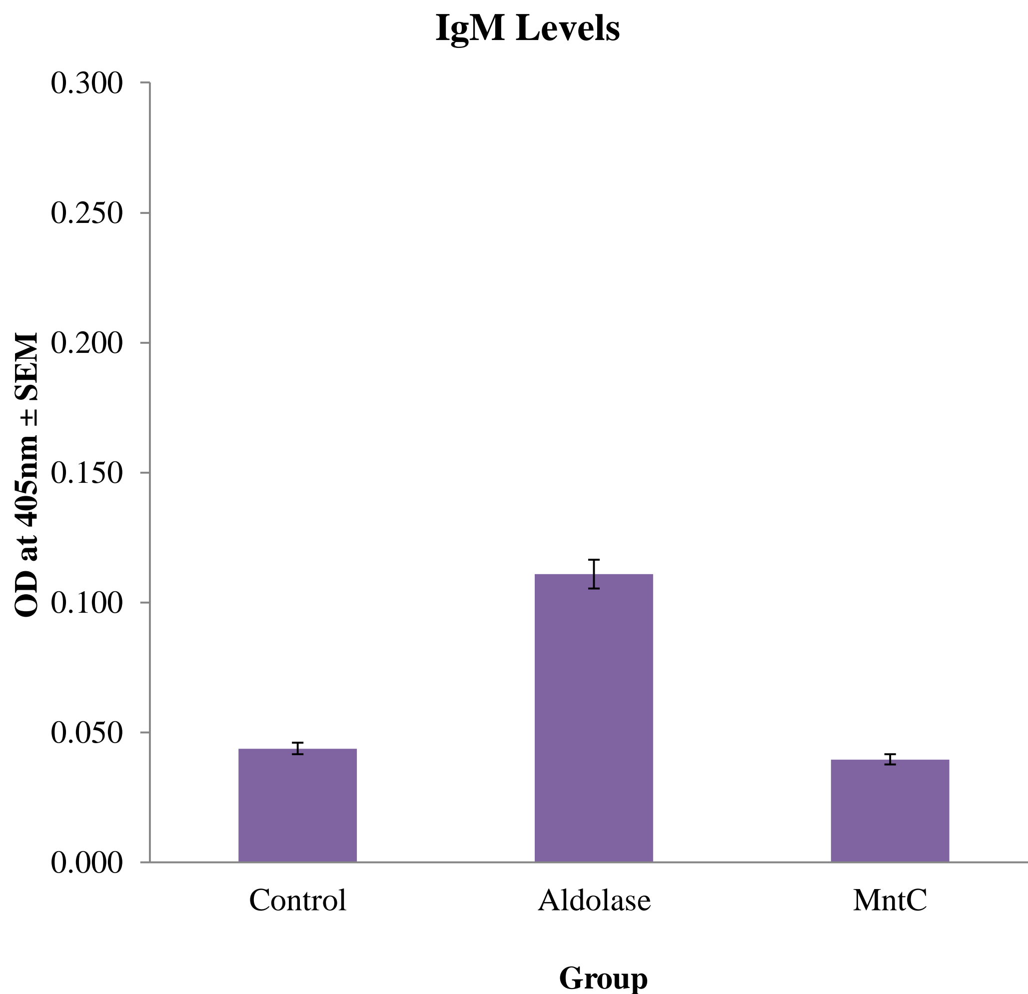
The IgG antibody titers of mice vaccinated using the same dosage regime were ~ 200 for with FBA- 1 and 3,200 for MntC respectively, using a cut-off point of 0.100 (Fig 1). The titer of MntC was significantly higher than that for FBA-1 ($p<0.02$).

Figure 1: Antibody titers of FBA-1 versus MntC versus Control mice



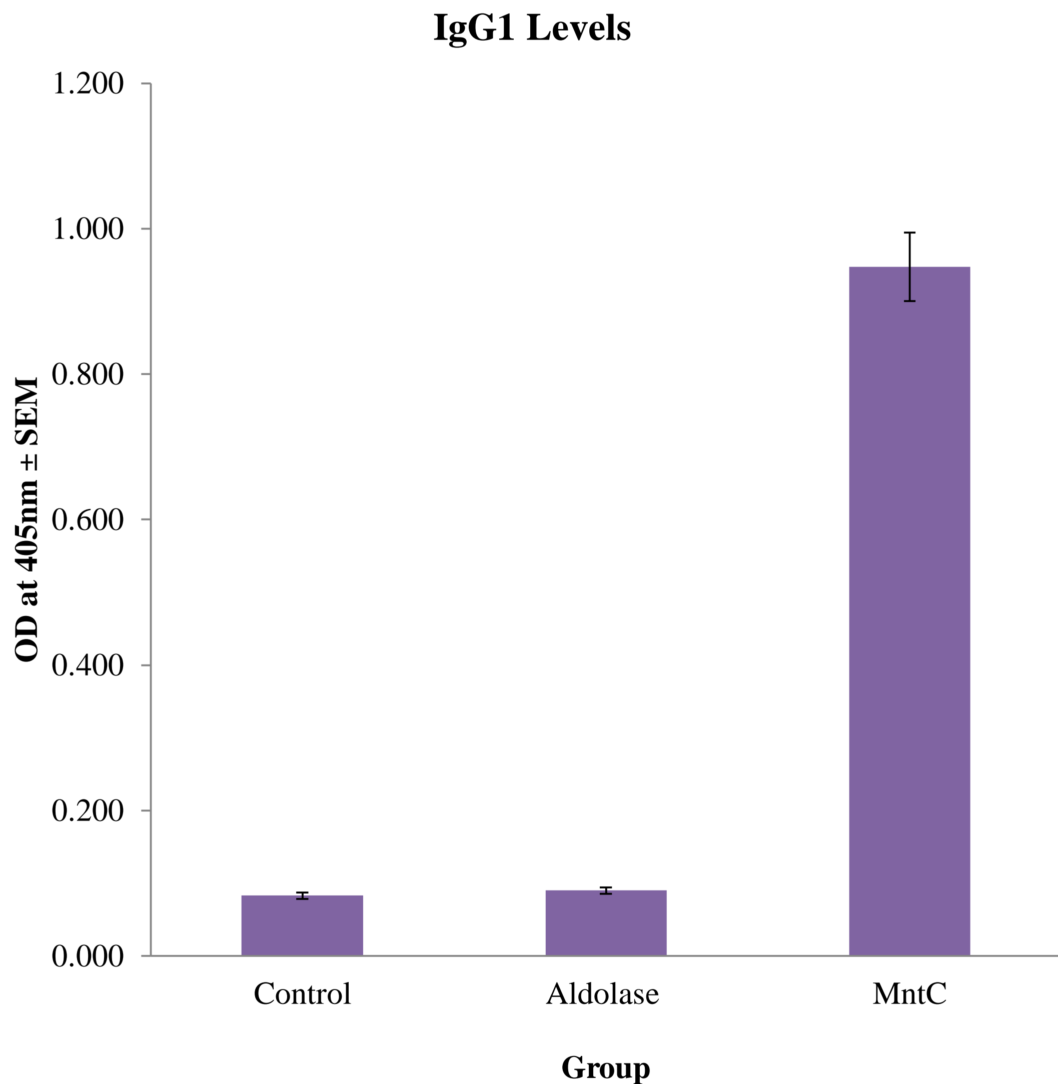
Immunisation of mice with FBA-1 resulted in the induction of only the IgM isotype whereas immunisation of mice with the MntC protein resulted in the production of IgG1 and IgG2a isotypes. The IgM isotype levels of mice immunised with FBA-1 were significantly greater (p-value < 0.005) than those of mice immunised with MntC or sham-immunised with PBS.

Figure 2: IgM levels of FBA-1, MntC versus Control mice



IgG1 levels were significantly higher in the antisera against MntC (Table 3, p-value < 0.002) as compared to the control. On the other hand, there was no IgG1 response in mice vaccinated with FBA-1 and sham-vaccinated control mice (Fig 3).

Figure 3: IgG1 levels of antisera against FBA-1, MntC versus the Control mice



IgG2a levels of sera were also significantly higher for MntC in comparison with that of FBA-1 and the sham-immunised control group of mice (p-value <0.002) as compared to the control (Fig 4). On the other hand, IgA was not induced against either FBA-1 or MntC (Figure 5).

Figure 4: IgG2a levels of antisera against FBA-1, MntC and Control mice

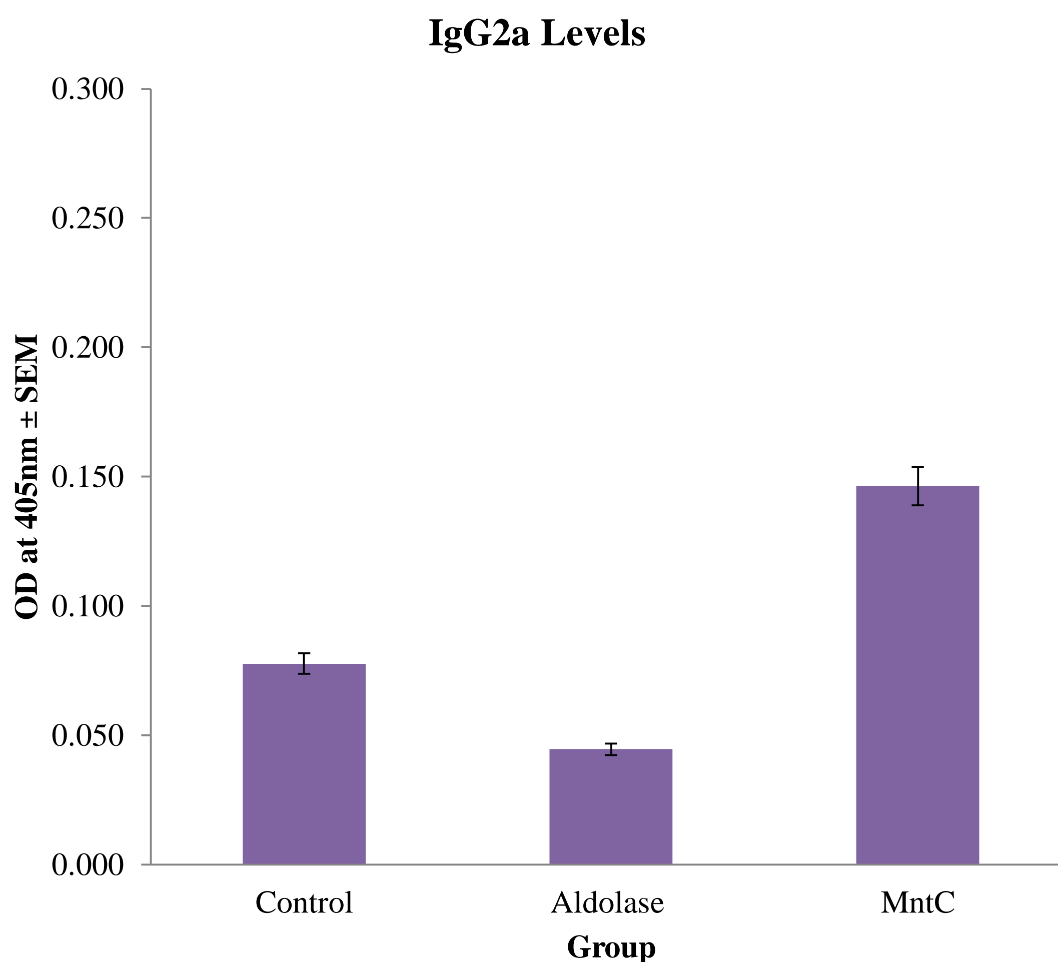
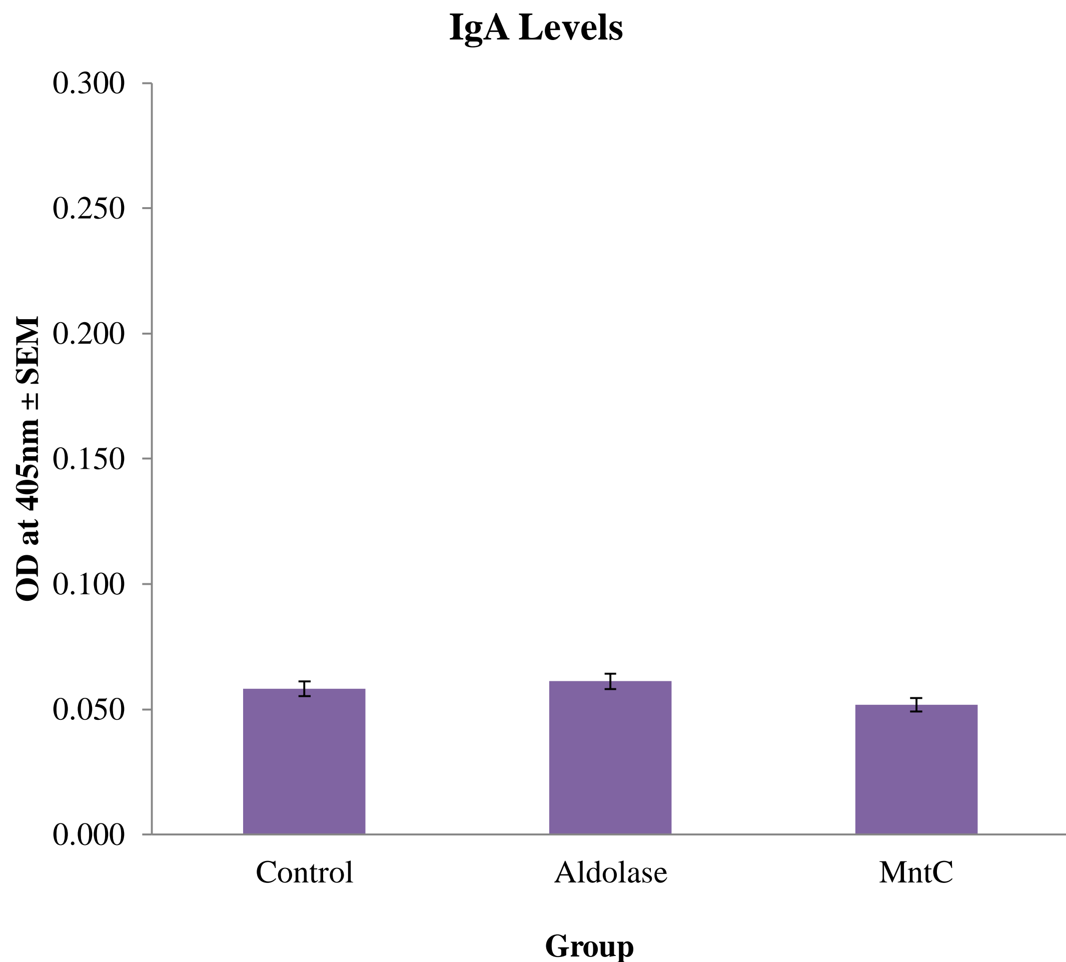


Figure 5: IgA levels of antisera against FBA-1, MntC and Control mice

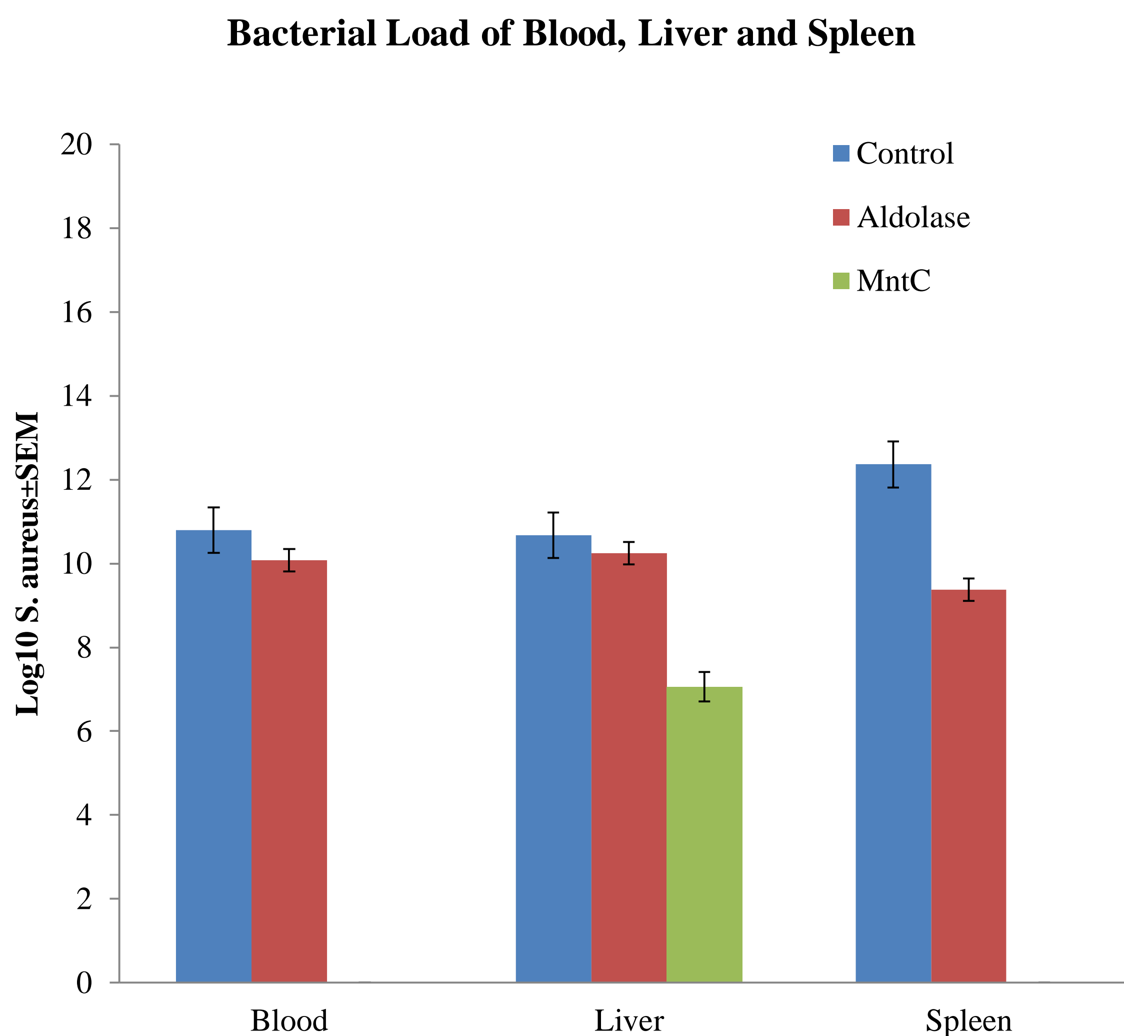


Blood and organ collection were serially diluted and plated out for bacterial load. Blood CFUs were calculated at 100µL whereas organ CFUs were caluclated per 100mg.

No *S. aureus* was detectable in the blood or spleen of mice vaccinated with MntC (Fig. 6). However, the load is significantly lower in the liver ($p<0.008$) than that

observed in FBA-1 vaccinated and the sham-vaccinated control mice. There was essentially no bacterial clearance from the blood or liver of mice vaccinated or sham-vaccinated with FBA-1 or PBS respectively. Clearance of *S. aureus* from the spleen of FBA-1 vaccinated mice was significantly higher ($p<0.007$) than that observed in the sham-vaccinated group showing a significant reduction (1.32-fold) in the bacterial load. Bacterial load was not significantly reduced in the mice administered with the anti-FBA-1 antibody with only a 1.04 fold reduction.

Fig 6: Bacterial load in the tissues of mice immunised with MntC versus FBA-1.



Discussion

Bacterial biofilms are a major concern in the treatment of persistent infections, with more than 80% of infections being difficult to treat because of biofilm formation. [10] No effective vaccine against infections caused by the versatile *S. aureus*, a pathogenic bacterium that easily adapts to its external environment is currently available. [11] Many different vaccine candidate antigens have been evaluated in laboratory animal models and also in humans in the search of an effective vaccine. [11-15]

More recently proteins produced by *S. aureus* have been shown to function as dual proteins such as MntC. MntC is a manganese transport protein that has been implicated as a microbial surface component recognizing adhesive matrix molecule (MSCRAMM). [8, 16] MntC is a cell surface protein that is expressed *in vivo* early in infection and is part of the Mnt complex. The Mnt complex comprises MntA, MntB and MntC making up an ABC transporter, or ATP binding cassette. [8, 16] The MntC is a lipoprotein that specifically binds to manganese whereas MntA is an ATP binding protein and MntB is an integral membrane transporting protein. [8]This investigation has confirmed, using the passive immunotherapy mouse model, the protective potential of MntC reported previously using the rat model (Anderson et al., 2012). [8, 16] However, antibodies against FBA-1, a recently identified biofilm-associated antigen, were unable to provide significant passive protective potential against *S. aureus* bacteraemia as judged by only 1.07 fold and 1.04 fold reductions in bacterial load in the blood and liver respectively. On the other hand, the CFUs of *S. aureus* were significantly reduced ($p<0.005$) in the spleen with a 1.32 fold reduction.

There are number of possible explanations for the poor performance of FAB-1 in protecting mice against acute bacteraemia. Expression of FBA-1 may not occur as early in infection as that reported for the MntC (Anderson et al., 2012). A second potential reason may be the comparatively inferior immunogenicity of the FBA-1 versus the MntC, either requiring higher doses for immunisation or a higher dose of the adjuvant. The MntC antigen of *S. pneumoniae* was previously reported to elicit a

protective immune response in mice, immunised with 25µg of protein injected intraperitoneally with 75µL of Inject Alum adjuvant, against challenge with the pathogen (Ling *et al*. 2004). The 3rd possible reason may be the sharing of a common epitope between the FBA-1 of *S. aureus* and mammals, despite the low level of observed similarity with protein sequence of *Homo sapiens*, a mammalian species. A 4th potential reason for the poor protective potential may be the induction of the antibody isotype IgM only which has a significantly shorter half life than the other antibody isotypes (IgG1 and IgG2a). Further studies are clearly warranted for testing of these hypotheses.

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Chapter 9

Waryah CB, Gogoi-Tiwari J, Wells K, & Mukkur T (2015) Development of a novel inhibition assay for identification of the most significant biofilm forming antigens of *Staphylococcus aureus.*

Manuscript Submitted

Development of a novel inhibition assay for identification of the most significant biofilm forming antigens of *Staphylococcus aureus*

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Running title: Biofilm assay for antigen significance in *S. aureus*

Key Words: biofilm, MSCRAMM, *Staphylococcus aureus*, antigen, antibody, novel detection method, MBEC assay

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Abstract

Biofilm formation is an important contributor to the virulence of bacterial pathogens due to promotion of persistence/colonization, persistent resistance to antibiotics and host's innate immune system. This study describes and validates an antibody-based inhibition assay for determination of the most significant virulence-associated antigens associated with biofilm formation. *Staphylococcus aureus* was used as a model pathogen for this investigation because it causes systemic and/or localised infections in both humans and animals. Using monoclonal or polyclonal antibodies against a multiplicity of accredited structural or secreted virulence antigens such as surface adhesins and exotoxins, it was confirmed that ClfA, FnBPA, SdrD, PNAG and α-toxin were the most significant antigens associated with biofilms of *S. aureus* as judged by 50% or greater inhibition of biofilm formation *in vitro* with specific antibodies. However, antibodies against accredited protective antigens viz., MntC, isdD, isdB and Bbp, were found to inhibit biofilm formation by only 20% indicating their minor contribution to biofilm formation. No inhibition of biofilm formation was observed with antibodies against select major super-antigenic toxins or immune evasion antigen, Protein A. The concept underpinning the described method has the potential for identification of biofilm-associated antigens of other bacterial pathogens.

Introduction

Staphylococcus aureus has a remarkably large array of virulence antigens which include surface-associated immune evading capsular polysaccharides, biofilm-associated Poly-N-acetyl glucosamine (PNAG), exotoxins and extracellular binding proteins, collectively known as microbial surface components recognizing adhesive matrix molecules, or MSCRAMM (Kropec et al., 2005). The major accredited exotoxins of significance include alpha toxin, toxic shock syndrome toxin (TSST) and multiple staphylococcal enterotoxins (SEs) (Verkaik et al., 2010) whereas the major MSCRAMM include Clumping factor A (ClfA), Protein A (Spa), fibronectin

binding protein A (FnBPA), iron-responsive surface-determinant A and B (IsdA, IsdB), serine aspartate repeat gene proteins D and E (SdrD, SdrE) and bone sialoprotein-binding protein (Bbp) (Foster et al., 2014).

Four major capsular phenotypes of *S. aureus* have been recognised (O'Riordan and Lee, 2004) thus far. Lack of relationship between encapsulation and biofilm formation has been reported previously (Babra et al., 2014). In this study, an immunological method that may be used to identify potential biofilm-associated antigens of *S. aureus in vitro* has been described. The identified biofilm-associated antigens could be used in the development of potentially effective conjugate vaccine formulations against infections caused by this *S. aureus*.

The MBEC™ (minimum biofilm eradication concentration) Assay, formerly named as the Calgary Biofilm Device, was developed as a biofilm growth device for bacterial pathogens (Ceri et al., 1999). The MBEC™ lid comprises of 96 pegs that corresponds with the wells of a standard 96 well microtiter plate. The MBEC assay is routinely used as an wet laboratory based platform for the determination of Minimum Inhibitory Concentration (MIC), a standard laboratory measurement for susceptibility testing (Harrison et al., 2005). In addition, the MBEC assays can be used for testing of susceptibility of pathogens to antibiotics, disinfectants, heavy metals as well as biofilm formation (http://www.innovotech.ca/products_mbec.php).

Numerous studies have confirmed that MIC may be different for free-floating versus biofilm bacterial cultures (Ceri et al., 1999; Babra et al., 2014). However, the MBEC assay has not thus far been adapted for determination of inhibition of biofilm formation by bacterial pathogens using specific antibodies. In this investigation, an antibody-mediated biofilm inhibition assay was developed for determination of the most significant virulence antigens of *S. aureus* potentially contributing to biofilm formation using the principle underpinning an indirect enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Antibodies used in this study are listed in Table 1, with their origin and source, and the highest titres/working dilutions used in the specific antibody-based biofilm inhibition assay.

Table 1: Antibodies towards several MSCRAMM and toxins obtained including their origin and working dilution factor.

Antibody	Target	Origin	Titer of aby	Source
Anti-*S.aureus* aby*	Whole cell	Rabbit	1 in 500	Abcam
Anti-α toxin aby	Toxin	Rabbit	1 in 50,000	Sigma Aldrich
Anti-ClfA aby	MSCRAMM	Mouse	1 in 5,000	Tim Foster
Anti-Spa aby	MSCRAMM	Goat	1 in 5,000	Abcam
Anti-FnBPa aby	MSCRAMM	Rabbit	1 in 2,000	Tim Foster
Anti-TSST aby	Toxin	Rabbit	1 in 5,000	Abcam
Anti-isdA aby	MSCRAMM	Mouse	1 in 5,000	Tim Foster
Anti-isdB aby	MSCRAMM	Mouse	1 in 5,000	Tim Foster
Anti-SdrD aby	MSCRAMM	Mouse	1 in 4,000	Tim Foster
Anti-SdrE aby	MSCRAMM	Mouse	1 in 3,000	Tim Foster
Anti-Bbp aby	MSCRAMM	Mouse	1 in 2,500	Tim Foster
Anti-SE aby	Toxin	Mouse	1 in 10,000	Abcam
Anti-PNAG aby	Biofilm	Goat	1 in 8	Gerald Pier

*Aby denoted antibody

Bacterial control strains

The quality control *Staphylococcus aureus* strain, a wound isolate, used in this investigation was ATCC® 29213™, a strong biofilm producer. In this investigation ATCC® 29213™ also served as the bacterial control for MSCRAMM viz., PNAG, ClfA, Spa, FnBPA, IsdA, IsdB, SdrD, SrdE and Bbp. A clinical strain in our laboratory collection, H23, was designated as the control for MntC after positive amplification with MntC primers (unpublished).

Controls for toxins were as follows: α toxin control was ATCC® 8096™, β toxin control was ATCC® 13565™ and TSST control was ATCC® 51651™. Two strains served as the control for the Staphylococcal Enterotoxins (SEs): ATCC® 13565 ™, purchased from ATCC and Strain Smith Diffuse, kindly donated by Professor Gerald Pier, Channing Laboratory, Brigham and Women's Hospital, Boston MA. ATCC® 13565 ™ was positive for SEA, SEC and SED whereas Smith Diffuse strain was positive for SEA, SEB and SEC.

Optimization of a PEG method

After confirming the working dilution factor for antibodies to be used in the peg-based assay using an adapted indirect ELISA assay (Fry et al., 2008), designated wells in a 96 well microtiter plate were filled with 200µL of antibody. The MBEC™ Biofilm Inoculator plate lid (Innovotech Inc) was placed on top of the antibody containing wells and placed on an orbital shaker, 80rpm, for 2hrs at 37ºC or overnight at 4ºC.

Staphylococcus aureus control strains were grown overnight in 2mL of NB on an orbital shaker (80rpm). Bacterial cells were centrifuged and washed twice in PBS. Cells were diluted in PBS to adjust the absorbance to 0.132 at OD_{600nm} (equivalent to approximately 10^8 CFU/mL) and placed on ice to prevent multiplication. Prior to incubation with the peg plate, the bacterial culture (0.132 at OD_{600nm}) was diluted tenfold (equivalent to 10^7 CFU/mL), in NB containing 1% glucose.

The MBEC plate lid was gently washed 3 times in chilled wash buffer. In a fresh 96 well microtiter plate, 200µL of 1:10 bacterial suspension was added to the corresponding antibody coated peg. The MBEC peg lid was placed on culture and incubated for 3hrs on an orbital shaker, 80rpm, at 37ºC to allow formation (or lack of formation) of biofilm on the peg.

Pegs were cut off the lid aseptically and washed three times in chilled sterile PBS (5mL/wash). After the final wash, the peg was placed in chilled 5mL of sterile PBS buffer on ice and subjection to gentle sonication at 47kHz ±6% in a water sonicator (Bransonic 1200 E4) for 20 mins to disrupt the peg-bound bacteria. Sonication was used to dislodge bacteria because no loss of viability *S. aureus* had been observed in standardisation of the method (data not shown). Pegs were immediately removed and discarded. The dislodged bacteria (100 µL) were aseptically spread-plated onto MH Agar plates, incubated overnight at 37ºC and CFUs determined. For every peg method, control groups without antibody were used for calculation of percentage reduction in bacterial colony counts. The CFUs for each control microorganisms in the biofilms of uncoated and antibody coated Pegs were determined in triplicate.

Results and Discussion

The concept underpinning the developed PEG method is direct inhibition of biofilm formation by specific antibodies against virulence factors of *S. aureus* using the MBEC™ Biofilm Inoculator plate. Using accredited ATCC controls against Staphylococcal toxins and MSCRAMM, biofilm inhibition was determined using corresponding specific antisera. Using the highest working dilution of antibodies, determined using indirect ELISA (Table 1), biofilm inhibition experiments were carried out to determine the most prevalent detectable virulence antigens associated with biofilms of *S. aureus*.

There have been numerous attempts to develop effective vaccines for prevention of infections caused by *S. aureus* in humans (Fattom et al., 2004; Kropec et al., 2005; Maira-Litran et al., 2005; Verdier et al., 2007; Bubeck Wardenburg and Schneewind, 2008; Huda et al., 2011; Pozzi et al., 2012) but none has claimed success in developing a universal vaccine thus far in achieving this goal for all infections caused by this pathogen (Lee, 2003 ; Schaffer and Lee, 2008; Pier, 2013). The basis of selection of different antigens for vaccine development in the formulation of conjugate vaccines has been their protective potential, judged mainly by reduction in bacterial loads in select organs of mice administered antigen-specific antibodies, and improvement in the opsonophagocytic killing indices of *S. aureus*. Given that most bacterial pathogens form biofilms, which in addition to promoting persistent antibiotic resistance (Babra et al., 2014), attenuate the effectiveness of the host's innate immune defences including inflammation (Archer et al., 2011; Thurlow et al., 2011; Babra et al., 2014), it is important to identify the antigens associated with biofilms of *S. aureus*.

The common method used for determining the association of a bacterial virulence antigen with biofilm formation is the generation of knockout mutants devoid of the target gene such that it loses the ability to form a biofilm (Caiazza and O'Toole, 2003; Anderson et al., 2012b; Pozzi et al., 2012). However, there are no reports that have determined the relatibe potential contribution of biofilm-asociated antigens of S. aureus. Furthermore, since this process of constructing knockout mutants can be

time consuming and unpredictable, the immunological assay described in this communication offers this opportunity.

O'Neill et al, (2007) reported that among MSSA isolates grown in media supplemented with 4% sodium chloride, PIA/PNAG production correlated with biofilm development. Our previous studies (Babra et al., 2014) confirmed these results. On the other hand, no PNAG production in MRSA isolates grown in the presence of either glucose or 4% NaCl was detected despite the fact that the *ica* operon was transcribed suggesting *ica*-independent mechanism presumably mediated by a protein adhesins. In this investigation, specific antibodies against the accredited virulence antigens inhibiting biofilm formation were found to range from 50 to ~80% (Table 2) using the antibody-based biofilm-inhibition assay. Specific anti-α toxin, anti-PNAG, anti-SdrD, anti-ClfA and anti-FnBPA antibodies inhibited biofilm formation by 77%, 69.8%, 57.6% and 52% respectively. Since specific anti-FnBPB antibodies, which have been reported to contribute to formation of biofilm by *S. aureus* (Geoghegan et al., 2013) were not available, its potential association with biofilm formation using the immunological assay could not be determined.

Table 2: A brief summary of antibody and the respective bacterial control with the percent reductions observed in the biofilm inhibition assay

Antibody	Bacterial control	CFU/mL** with Antibody	CFU/mL** without Antibody	Percent Reduction	P-value
Anti-*S. aureus* aby	ATCC® 29213™	$3.80 \pm 0.28 \times 10^{2}$	$1.11 \pm 0.107 \times 10^{3}$	65.9%	0.011
Anti-α toxin aby	ATCC® 8096™	$1.0 \pm 0.04 \times 10^{3}$	$4.37 \pm 0.29 \times 10^{3}$	77%	0.006
Anti-ClfA aby	ATCC® 29213™	$1.92 \pm 0.058 \times 10^{3}$	$4.53 \pm 0.20 \times 10^{3}$	57.6%	0.003
Anti-Spa aby	ATCC® 51651™	$2.96 \pm 0.083 \times 10^{3}$	$2.66 \pm 0.131 \times 10^{3}$	NIL	N/A
Anti-FnBPa aby	ATCC® 29213™	$1.2 \pm 0.15 \times 10^{2}$	$2.5 \pm 0.29 \times 10^{2}$	52%	0.049
Anti-TSST aby	ATCC® 51651™	$3.65 \pm 0.074 \times 10^{3}$	$2.66 \pm 0.131 \times 10^{3}$	NIL	N/A
Anti-isdA aby	ATCC® 29213™	$8.77 \pm 0.74 \times 10^{2}$	$1.19 \pm 0.037 \times 10^{3}$	26.5%	0.080
Anti-isdB aby	ATCC® 29213™	$5.0 \pm 0.14 \times 10^{2}$	$6.5 \pm 0.29 \times 10^{2}$	22.3%	0.063
Anti-SdrD aby	ATCC® 29213™	$2.6 \pm 0.60 \times 10^{2}$	$6.5 \pm 0.29 \times 10^{2}$	60%	0.009
Anti-SdrE aby	ATCC® 29213™	$2.4 \pm 0.29 \times 10^{2}$	$3.0 \pm 0.57 \times 10^{2}$	20%	0.514
Anti-Bbp aby	ATCC® 29213™	$3.92 \pm 0.380 \times 10^{3}$	$4.53 \pm 0.020 \times 10^{3}$	17.9%	0.237
Anti-SE aby (Positive SEs)	ATCC® 13565 ™ (SEA, SEC, SED)	$1.08 \pm 0.035 \times 10^{3}$	$1.19 \pm 0.142 \times 10^{3}$	9.2%	0.562

	Strain Smith Diffuse* (SEA, SEB, SEC)	9.5±0.06 x10^2	1.33±0.32 x10^3	28.8%	0.005
Anti-PNAG aby	ATCC® 29213™	2.35±0.003 x10^3	7.78±0.004 x10^3	69.8%	0.000
Anti-MntC aby	Human strain S23	1.84±0.080 x10^3	2.68±0.092 x10^3	31.3%	0.021
Anti-Aldolase aby	Human strain S23	2.0±0.197 x10^3	2.69±0.092 x10^3	23.9%	0.135

***Strain Smith Diffuse has been previously identified as positive for SEA, SEB and SEC**

****Average CFU/mL (n=3)**

Examining the relative prevalence of different virulence genes, we found that genes encoding the MSCRAMM Spa, ClfA, ClfB, SdrE, SdrD, IsdA, IsdB and MnTc were detected in greater than 90% of the isolates whereas those producing FnBPA accounted for only 6.5% of the total number of isolates examined (unpublished). Genes for α-toxin were also detected in greater than 90% of the isolates. Interestingly, antibodies against Protein A showed no association with biofilm formation. While this may have been due to the difference in the *spa* type of the strain used to raise polyclonal antibodies by the supplier, Abcam, the level of *spa* expression has been reported to have no detectable effect on non-specific killing in opsonophagocytic antibody assay (OPA) (Nanra et al., 2012). However, despite the reported protective potential of surface Protein A (Yi et al., 2012), no contribution of this immune evasion molecule to biofilm formation was observed. While in contrast to the finding reported by Merino et al (2009), our finding supports the recent report by Foulston et al, (2014) that protein A and fibrinogen-binding proteins A did not contribute significantly to biofilm formation. It would however be interesting to examine the inhibitory effect of specific anti-FnBPB antibodies on biofilm formation by *S. aureus*.

The relatively insignificant contribution of MntC to biofilm formation found in this study by the Peg assay supports the speculation stated by Salazar et al (2014) that “the protective role played by MntC in animal models may have been due to impairment of its adhesive properties” despite its multifunctional role as an ion-scavenging and binding properties for ECM and potential binding to proteins in the coagulation cascade in the host (Anderson et al., 2012a). The observation that no apparent contribution of enterotoxin A, C and D was does not rule out potential contribution by enterotoxin B and warrants determination using specific anti-SEB antibodies.

Previously published research has demonstrated association of individual virulence antigens with biofilm formation (Deivanayagam et al., 2000; Foster, 2002; Caiazza and O'Toole, 2003; Kropec et al., 2005; Kim et al., 2010) but not on their comparative potential contribution to biofilm formation as determined using the

immunological assay described in this communication. The concept underpinning this method may also be applicable for determining the comparative contribution of different virulence factors/antigens of other human and animal bacterial pathogens.

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Chapter 10 – Conclusion

Staphylococcus aureus is the most common causative agent of invasive staphylococcal infections and is commonly referred to as “Golden Staph” (Lyon & Skurray, 1987). This facultative anaerobic bacterium has also been isolated from other infections such as toxic shock syndrome, endocarditis and septicaemia (O'Riordan & Lee, 2004, Skurnik *et al.*, 2010). It has been isolated from nosocomial infections, which often occur in the form of infection of permanent prosthetic implants, contact lenses and urinary tract infections, among many others (Cramton *et al.*, 1999, Ando *et al.*, 2004, Anderson *et al.*, 2012).

Because of a multitude of clinical manifestations/disease syndromes caused by multiple antibiotic resistant *S. aureus*, particularly pneumonia and sepsis in neonates, it is urgent to develop an effective prophylactic vaccine against *S. aureus* that can be used in pregnant mothers to impart protection to the neonate via colostrum and milk, and in elderly patients prior to any surgical intervention in hospitals worldwide. Given that majority of staphylococcal infections (80%) are biofilm-associated (Harro *et al.*, 2010, Anderson *et al.*, 2012) and biofilm-associated pathogens are 50 to 500 times more resistant with potential to lead to chronic infection, it is quite plausible that biofilm-associated *S. aureus* has different proteome than its planktonic phase.

This study was designed to explore the potential of previously unknown virulence and/or biofilm-associated antigens, particularly surface-associated proteins, leading to development and persistence of resistance to antibiotics. It is therefore important to determine not only the novel antigens associated with biofilm formation but also contribution of the already accredited surface antigens, termed as MSCRAMM, during biofilm formation.

This investigation revealed substantial association between biofilm formation and the presence of PNAG (Babra *et al.*, 2013). The association was substantial albeit not

absolute indicating the presence of additional potential MSCRAMM contributing or associated with the formation of biofilm by *S. aureus* There was also a strong association between biofilm formation of MSSA strains and persistence of antibiotic resistance (Cernohorska, 2010, Babra *et al.*, 2013, Babra *et al.*, 2013) with a higher rate of resistance developed in the biofilm-embedded state, which is most likely a contributing factor to treatment failures recorded for *S. aureus* infections. Resistance was maintained up to day 30 following subculturing of the isolates, suggesting serious consideration be given to investigate the antibiograms for *S. aureus* from infected patients in both biofilm and planktonic cultures prior to the commencement of treatment with antibiotics. Although the ability of biofilm to resist to treatment with antibiotics has long been acknowledged however this study reported the persistence of antibiotic resistance with formation of biofilm (Babra *et al.*, 2013).

Extensive research has been carried on capsular polysaccharides (O'Riordan & Lee, 2004) of *S. aureus* as immune evasion molecule and its use in the formulation of conjugate vaccines against infections caused by *S. aureus* in immune-competent and immune-compromised subjects including animals and humans (Fattom *et al.*, 1996, Han *et al.*, 2000, Robbins *et al.*, 2004, Kampen *et al.*, 2005, Nanra *et al.*, 2012). However, their role in biofilm formation had not been explored. Using well-established, independent *in vitro* biofilm analysis methods – TCP and Congo red agar methods (Babra *et al.*, 2013) and CP serotyping and genotyping, it was discovered and reported (Waryah *et al.*, 2014) that there is no association between the production of capsule and biofilm formation. In addition to this discovery, an improved optimized modified TCP method, with defined cut off points used for biofilm formation, was developed (Babra *et al.*, 2013)

A comparison of the serotyping method, developed using bovine mastitis isolates in this laboratory by Gogoi-Tiwari *et al.*, 2014, Australian Veterinary Journal, in press), revealed that serological typing as a better method for presence of capsule and the somatic polysaccharide 336 phenotypes than genotyping. In Western Australia, the capsular phenotypes 5 and 8 were the predominant capsular types *S. aureus* human clinical and student *S. aureus* isolates. However, there were a number of isolates that were found to be encapsulated determined by the use of an improved Maneval's

capsular staining method incorporating the use of a sucrose gradient in the method to remove the non-encapsulated and hence non-typeable *S. aureus* isolates (Waryah *et al.*, 2014).

Previous to investigations presented in this thesis, it was generally accepted that there were only 4 capsular types of *S. aureus*, the remaining capsular types being labelled as non-typeable or serotype 336 (Nanra *et al.*, 2012). One unexpected discovery found in this investigation was the prevalence of additional capsular types observed by the presence of capsule on non-CP1, CP2, CP5, CP8 or serotype 336 isolates. Due to time constraints, the scope of this thesis was unable to accommodate exploration of this interesting finding. One limitation of the capsular typing study was the lack of primers against type 336. However given the high specificity of the serotyping analysis, this limitation was easily overcome. Future investigation in validity of serotype 336 as a somatic non-capsular antigen is also warranted.

Using a combination of genotyping and serotyping methods, 26 virulence factors including MSCRAMM and toxins, were found to be distribution among the available or collected *S. aureus* isolates. Diversity of the strains, determined using RAPD analysis, revealed the distribution of virulence genes to be diverse with genes encoding MSCRAMM Spa, ClfA, ClfB, SdrE, SdrD, IsdA and IsdB dominant in 90% of isolates. Genes encoding α-toxin were detected in greater than 90% of the isolates followed by β-toxin and SEG enterotoxin detected in 50-60% of the isolates.

Many investigations that have concentrated on finding a single target for development of a vaccine against many bacterial infections including those caused by *S. aureus* (Middleton, 2008, Huda *et al.*, 2011, Sharma *et al.*, 2011, Shahrooei *et al.*, 2012, Jiang *et al.*, 2014). On the other hand, many others have targeted development of vaccines using a combination of inactivated virulence antigens as conjugate vaccines involving linkage with surface-associated polysaccharide antigens, including PNAG, but without gaining knowledge about their contribution to formation of biofilms (Maira-Litran *et al.*, 2005, Pozzi *et al.*, 2012). Although PNAG has already been used in the formulation of conjugate vaccines using select MSCRAMM antigens, my project is the first one to report the significant biofilm-

associated virulence antigens using a novel peg-based technique (Waryah, submitted for publication to the Journal of Medical Microbiology). This study has indicated the importance of developing conjugate cocktail vaccines based on the contributions of the major MSCRAMM to biofilm formation.

In addition to identifying the potential antigens contributing to biofilm formation by *S. aureus*, this study also investigated the effect of dispersal of the biofilms using biofilm-degrading enzymes on the antimicrobial efficacy of antibiotics *in vitro*, using tobramycin as a model antibiotic. Extracellular bacterial DNA (eDNA) and the surface-associated PNAG have been demonstrated to play important roles in biofilm formation by *S. aureus* (Haaber *et al.*, 2012, Lister & Horswill, 2014). When used as singular enzymes, the antimicrobial efficacy increased 8780 fold for DNAse I and 7500 fold for dispersin-treated biofilms. However, when combined together for treatment of the biofilm, a significant reduction in the antibiotic efficacy of tobramycin was observed (manuscript submitted for publication). This study demonstrated that using Tobramycin with DNase I or Dispersin B alone enhances the bactericidal activity of the antibiotic. Previously thought to be a weak disruptor of *S. aureus* biofilms, Dispersin B with Tobramycin was effective in dispersing the biofilms enough to significantly enhance the antimicrobial efficacy of tobramycin, an antibiotic widely used in patients suffering from cystic fibrosis (Khan *et al.*, 1995, Sawicki *et al.*, 2012, Trapnell *et al.*, 2012). One limitation of this study is the testing of only Tobramycin. However, the principle underpinning the use of biofilm-disrupting agents to enhance the efficacy of antibiotics has been demonstrated. The exact treatment strategies for different bacterial infections for different persistent microbial infections caused by different pathogens would need to be worked out *in vitro* prior to their assessment *in vivo*. The mechanisms underpinning the observed mutual inhibition between these 2 biofilm-degrading enzymes were not determined because of time constraints. However, the evaluation effect of coating medical devices with the biofilm degrading enzymes for prevention of biofilm formation, are highly warranted.

Staphylococcus aureus grown under the stressful iron-deprivation, revealed the presence of a unique over-produced protein band in the biofilm of this nosocomial

pathogen. The purified band, 33kDa in molecular mass, was identified as Fructose-bisphosphate aldolase Class 1 (FBA-1), which is also a moonlighting protein, by mass spectrometry. FBA Class 1 was previously reported to be present in the planktonic *S. aureus* cells but without any association with the biofilms.

Using the antibodies against FBA-1 and MntC in SPF Balb/c mice, passive protective potential of these antigen-specific sera was compared using the acute murine bacteraemia model. It is hypothesised that the better passive protective potential of anti-MntC sera may be either associated with the short half-life of the IgM versus the longer half-life of the IgG isotypes (IgG1 and IgG2a), need to use a higher dose, adjustment of the vaccine formulation to reflect a higher content of the adjuvant as has been reported for a *S. pneumoniae* antigen (Ling *et al.*, 2004) or the sharing of an epitope of FBA-1 with the mammalian counterpart despite a low level of similarity.

Future direction for validating the role of FBA-1 in establishment by biofilm formation and virulence should involve using the following approaches:

1. Determining the cross-reactivity of the *S. aureus* FBA-1 with the mouse and/or human FBA-1 serologically.

   Studies carried out on the immunogenicity and protective potential that are presented in this thesis, relied on the protein sequence homology data. If serological cross-reactivity of the *S. aureus* FBA-1 with mouse or human aldolase is discovered, which is highly unlikely, it will no longer be a candidate for vaccine development or a target for discovery of new antibiotics. Furthermore, the validity of determining the similarity/homology between 2 molecules using the protein sequence data will be under challenge.

   If no cross-reactivity is discovered as is expected, the approaches outlined below, should be pursued for confirming and/or validating the role of FBA-1 in biofilm formation and virulence of *S. aureus*. Using the novel peg-based antibody inhibition method described in **Chapter 9**, it has already been determined that both the FBA-1 and MntC of *S. aureus* form biofilms to the

same extent. However, MntC molecule has been shown to be expressed early in the infection cycle, which information is not available for the FBA-1 of *S. aureus*, hence the 2nd future direction.

2. Determining the phase at which the gene for FBA-1 is expressed in the invasive infection cycle of *S. aureus in vivo*: If FBA-1 is not produced in the early phase of the infection cycle, acute murine bacteraemia model, used by many research laboratories could not be considered as a suitable model for evaluation of its protective potential.

3. Determining the effect of lowering the levels of FBA-1 by regulating its production using anhydrotetracycline (Puckett *et al.*, 2014): Virulence of *S. aureus* grown under these conditions should be reduced assuming that FBA-1 is produced early in the infection cycle of *S. aureus* bacteraemia.

4. Determining the effect of deletion of the gene encoding FBA-1 on the virulence of *S. aureus in vivo* as has been reported for *Mycobacterium tuberculosis* (Puckett *et al.*, 2014). Deletion of FBA-1 of *S. aureus* should reduce its virulence permitting mice to live longer after challenge with the wild type.

5. Evaluating the dose response of recombinant FBA-1 of *S. aureus* with variable quantities of adjuvants with the aim of producing high-titred specific antisera containing IgG1 and IgG2 isotypes: This may involve evaluation of different types of traditional and other adjuvant formulations that have been reviewed by commercial companies such as Invivogen (http://www.invivogen.com/review-vaccine-adjuvants) and also some research laboratories (Mohan *et al.*, 2013, Bergmann-Leitner & Leitner, 2014).

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Appendix 1

Raw Data

Additional Information

Chapter 3

For biofilm formation, bacteria were grown in 96 well microtitre plates. Fig 1: Example of biofilm production *in vitro*

From left to right: weak, medium and strong biofilm producers

Table 1: Tissue Culture plate readings at 600nm using the Crystal Violet method

Strain	OD_{600nm}	Strain	OD_{600nm}	Strain	OD_{600nm}	Strain	OD_{600nm}
S1	0.723	**S11**	1.319	**S20**	1.172	**H6**	1.384
S2	1.328	**S12**	0.564	**S21**	1.271	**H7**	1.297
S3	1.133	**S13**	1.439	**S23**	1.439	**H8**	1.088
S4	1.048	**S14**	1.088	**H1**	1.388	**H9**	1.434
S5	1.098	**S15**	0.818	**H2**	1.119	**H10**	1.476
S6	0.893	**S16**	1.225	**H3**	1.468	**H11**	1.514
S7	1.115	**S18**	1.206	**H4**	1.167	**H12**	1.358
S9	1.445	**S19**	1.228	**H5**	1.435	**ATCC 29213**	1.288

Cut off was OD_{600nm} 0.120. Strains with a cut off of OD_{600nm} ≤0.480 were considered weak. OD values ≤0.720 were moderate biofilm formers and OD values >0.721 were considered strong biofilm forming bacteria

Fig 2: Production of a red pigment on Congo Red Agar plates indicates weak slime producers and production of black indicates strong slime producers

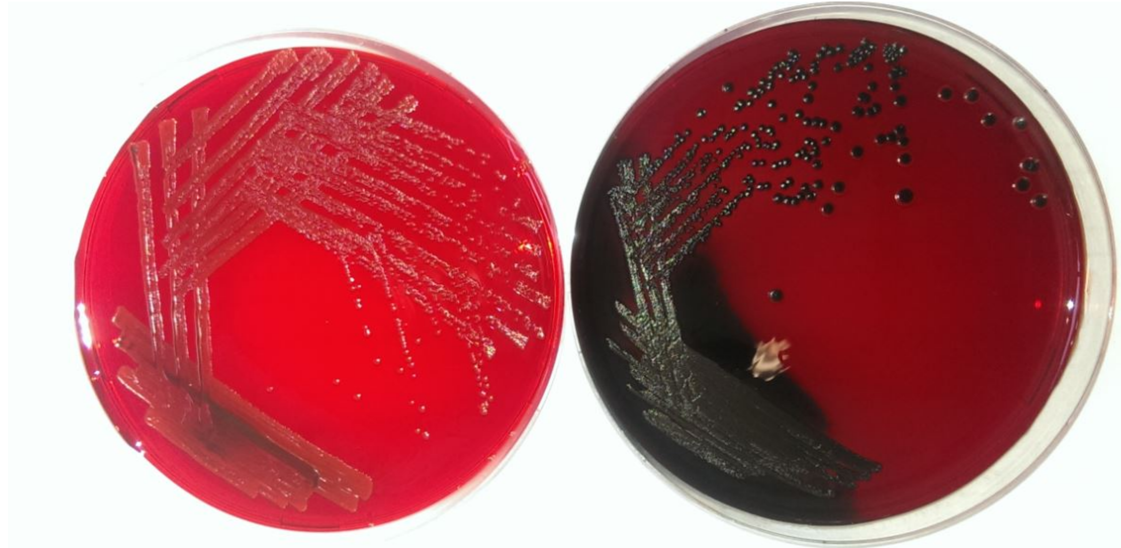


Table 2: Congo red agar plate colour observation after 72hr

Strain	Colour	Strain	Colour
S1	Red	**S11**	Red
S2	Red	**S12**	Red
S3	Black	**S13**	Dark red
S4	Black	**S14**	Red
S5	Black	**S15**	Dark red
S6	Black	**S16**	Dark red
S7	Red	**S18**	Black
S9	Black	**S19**	Dark red
Strain	**Colour**	**Strain**	**Colour**
S20	Dark red	**H6**	Dark red
S21	Dark red	**H7**	Black
S23	Red	**H8**	Red
H1	Red	**H9**	Black
H2	Red	**H10**	Black
H3	Black	**H11**	Dark red
H4	Black	**H12**	Red
H5	Black	**ATCC 29213**	Black

Fig 3: Control strains USA LAC (CP negative) and USA 400 MW2 (CP8) were positive for methicillin resistance identified on both Chromogenic MRSA ID plates and *mecA* amplification

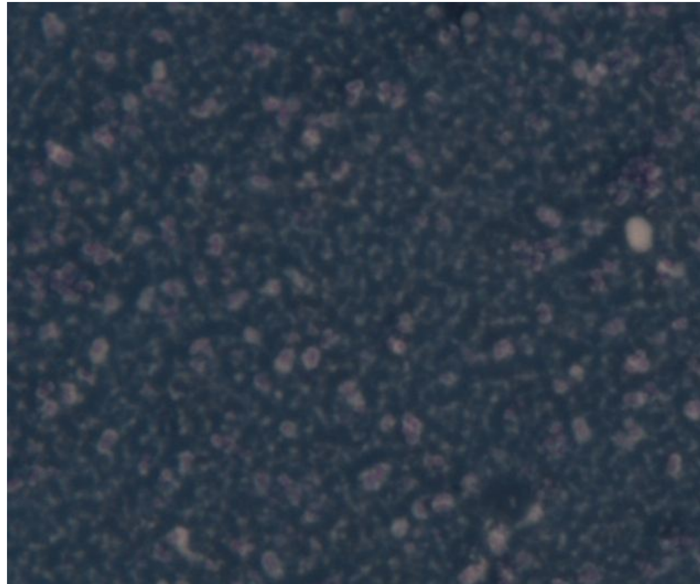
Table 3: Raw data comparing the capsular genotyping and serotyping observations

Sl. No	*S aureus* human strain	Detection of capsular genotype by PCR method				Detection of capsular serotype by Slide Agglutination test			
		CP1	CP2	CP5	CP8	CP1	CP2	CP5	CP8
1	S.aur 1	–	–	–	–	–	–	–	–
2	S.aur 2	–	–	–	✓	–	–	–	✓
3	S.aur 3	–	–	✓	–	–	–	✓	–
4	S.aur 4	–	–	–	✓	–	–	–	✓
5	S.aur 5	–	–	–	–	–	–	–	-
6	S.aur 6	–	–	✓	–	–	–	✓	–
7	S.aur 7	–	–	✓	–	–	–	✓	–
8	S.aur 9	–	–	✓	–	–	–	✓	–
9	S.aur 11*	–	–	✓	✓	–	–	-	✓
10	S.aur 12	–	–	✓	–	–	–	✓	–
11	S.aur 13	–	–	–	✓	–	–	–	✓
12	S.aur 14	–	–	–	✓	–	–	–	✓
13	S.aur 15	–	–	–	✓	–	–	–	✓
14	S.aur 16	–	–	–	✓	–	–	–	✓
15	S.aur 18	–	–	✓	–	–	–	✓	–
16	S.aur 19	–	–	✓	–	–	–	✓	–
17	S.aur 20	–	–	✓	–	–	–	✓	–
18	S.aur 21	–	–	✓	–	–	–	✓	–
19	S.aur 23	–	–	–	–	–	–	–	–
20	H1 - 769199	–	–	–	–	–	–	–	–
21	H2 - FH	–	–	–	–	–	–	–	–
22	H3 - 718972	–	–	–	✓	–	–	–	✓
23	H4 - Paul C Ear	–	–	–	✓	–	–	–	✓
24	H5 - 13188622	–	–	–	–	–	–	–	✓
25	H6 - Maria	–	–	✓	–	–	–	✓	–
26	H7 - Mel O	–	–	✓	–	–	–	✓	–
27	H8 - 28062009	–	–	–	✓	–	–	–	✓
28	H9 - 080989	–	–	–	✓	–	–	–	✓
29	H10 - 38911557	–	–	–	✓	–	–	–	✓
30	H11 - 9555100	–	–	–	✓	–	–	–	✓
31	H12 - 13965121	–	–	–	✓	–	–	–	✓
32	ATCC 29213	–	–	✓	–	–	–	✓	–

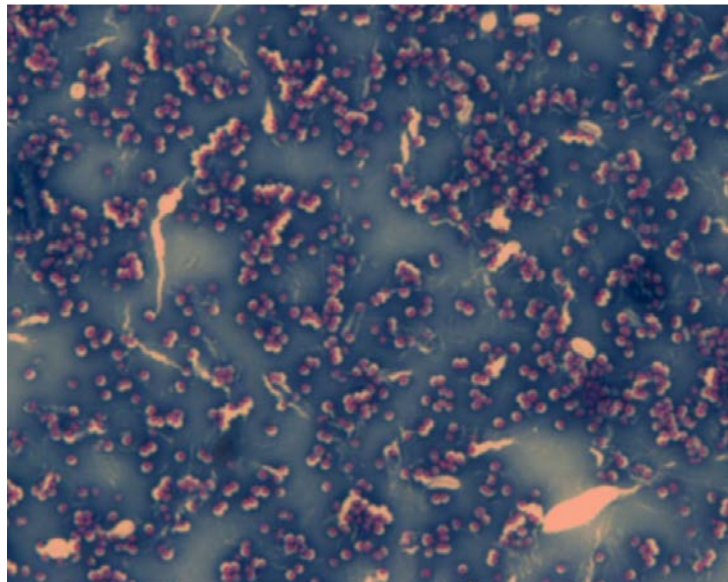
*Strain found positive by genotyping for CP5 and CP8 but was confirmed as only CP8 positive using serotyping

Figure 4 (a) and (b): A comparison of the improved Muir's Mordant method. Figure (a) shows a view of *S, aureus* stained with the original Muir's Mordant Method under the microscope at 100X. It is difficult to see the clear outline of the capsule (white halo around purple/red stained bacteria). Large deposits of background debris can be noticed in the slide. Figure (b) shows a view of *S. aureus* stained with the improved Muir's Mordant Method under the microscope at 100X. Only a few stain deposits, with essentially no cell debris, are visible.

(a)



(b)***



*** Picture showing the distinct capsule was selected as the front cover image for Journal of Medical Microbiology

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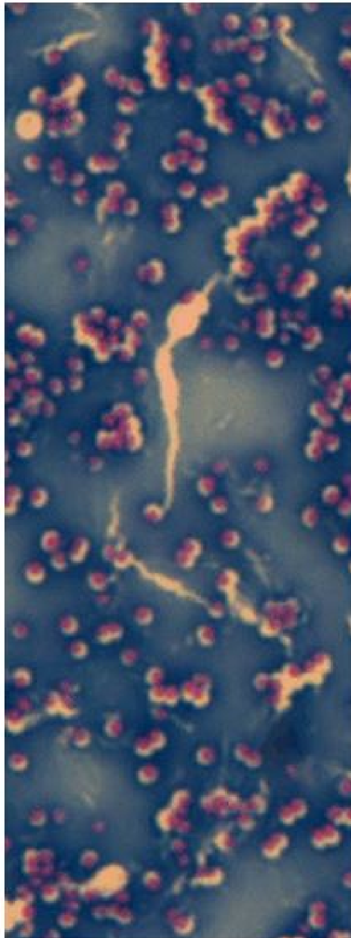
Cover image



Front cover illustration

Image(s) courtesy of Charlene Babra Waryah *et al.*, 2014 See the paper titled 'Serological versus molecular typing of surface-associated immune evading polysaccharide antigens - based phenotypes of Staphylococcus aureus' (doi:10.1099/jmm.0.077024-0) by Waryah *et al.* in the next issue for further information. Nontypeable *Staphylococcus aureus* isolate displaying a capsule using modified Maneval's capsule staining method.

Table 4: Raw data analysis of the Staphylococcal enterotoxin typing by PCR and serological kits

Genotyping					Serotyping			Serotyping			
					Tecra Staph Enterotoxin kit**			SET_RPLA toxin kit			
Strain No	SE A	SE B	SE C	SE D	Abs $OD_{490\pm10nm}$	Colour code		SE A	SE B	SE C	SE D
1	–	–	–	–	0.152	1	✓	–	–	–	–
2	✓	–	–	–	3.317	3	–	–	–	–	–
3	–	–	–	–	1.575	3	✓	–	–	–	–
4	–	–	–	–	3.242	4	✓	–	–	–	–
5	–	–	–	–	0.311	2	✓	–	–	–	–
6	✓	–	–	–	0.653	3	✓	–	–	–	–
7	✓	✓	–	–	0.134	1	–	–	–	–	–
9	✓	–	–	–	1.923	4	✓	✓	–	–	–
11	✓	–	–	–	1.547	4	✓	✓	–	–	–
12	✓	✓	–	–	3.252	5	✓	–	✓	–	–
13	–	–	–	–	0.457	2	✓	–	–	–	–
14	–	–	–	–	0.170	1	–	–	–	–	–
15	–	–	–	–	0.170	1	–	–	–	–	–
16	–	–	–	–	0.177	1	–	–	–	–	–
18	–	✓	–	–	0.234	2	✓	–	–	–	–
19	–	✓	–	–	0.208	2	✓	–	–	–	–
20	–	–	–	–	0.143	1	–	–	–	–	–
21	–	✓	–	–	0.140	1	–	–	–	–	–
23	–	–	–	–	0.168	1	–	–	–	–	–
H1	–	✓	–	–	3.244	5	✓	–	–	–	–
H2	–	–	–	–	0.111	1	–	–	–	–	–
H3	–	–	–	–	0.358	2	✓	–	–	–	–
H4	–	–	✓	–	0.234	5	✓	–	–	✓	–
H5	–	–	✓	–	0.857	2	✓	–	–	–	–
H6	–	–	–	–	0.273	1	✓	–	–	–	–
H7	–	–	–	–	0.115	1	–	–	–	–	–
H8	–	–	–	–	0.146	1	–	–	–	–	–
H9	–	–	✓	–	3.228	5	✓	–	–	✓	–
H10	✓	–	–	–	2.770	4	✓	✓	–	–	–
H11	✓	–	–	–	0.108	2	–	–	–	–	–
H12	–	–	–	–	0.133	1	–	–	–	–	–

**Kit was used in the study but not published

Table 5: Further analysis for comparison with the genotyping and serotyping against SEA, SEB, SEC and SED

SEA			**SEB**			**SEC**		
	Geno typing	Sero typing		Geno typing	Sero typing		Geno typing	Sero typing
S.aur2	✓	–	S.aur7	✓	–	H4	✓	✓
S.aur6	✓	–	S.aur12	✓	✓	H5	✓	–
S.aur7	✓	–	S.aur18	✓	–	H9	✓	✓
S.aur9	✓	✓	S.aur19	✓	–	SED: No positives		
S.aur11	✓	✓	S.aur21	✓	–	SEA: Only 2 were positive using both methods SEB: None of the strains were positive using both methods SEC: Only 2 were positive for both		
S.aur12	✓	–	H1	✓	–			
H10	✓	✓	– indicates a negative result ✓ indicates a positive result					
H11	✓	–						

Table 6: Binary data obtained from RAPD PCT analysis used to create the dendrogram

Primer C				
Isolate	Band 1	Band 2	Band 3	Band 4
1	0	0	1	1
2	0	1	1	1
3	0	1	1	0
4	0	1	1	1
5	0	1	1	1
6	0	1	1	1
7	0	1	1	0
9	0	1	1	1
11	0	1	1	1
12	0	1	1	1
13	0	1	1	1
14	0	1	1	1
15	0	1	1	1
16	0	1	1	1
18	0	1	1	1
19	0	1	1	1
20	0	1	1	0
21	0	1	0	1
23	0	1	0	1
H1	0	0	0	1
H2	0	0	0	1
H3	0	1	1	0
H4	0	1	1	1
H5	0	0	0	1
H6	0	0	1	0
H7	0	0	1	0
H8	0	0	1	0
H9	0	1	1	0
H10	0	0	1	1
H11	1	0	1	1
H12	0	0	1	0
SBP	0	0	1	0
CP1	0	0	1	1
CP2	0	0	1	0
CP5	0	0	1	0
CP8	0	0	1	1
LAC	0	0	1	0
51651	0	1	1	0
13565	0	1	1	0
49775	0	0	1	0
8096	0	0	1	1
T336	0	1	1	0

OPA13			
Isolate	Band 1	Band 2	Band 3
1	0	0	1
2	0	0	1
3	0	0	0
4	0	0	1
5	0	0	1
6	0	1	1
7	0	1	1
9	0	1	1
11	0	1	1
12	0	1	1
13	0	0	1
14	0	1	1
15	0	0	1
16	0	0	1
18	0	1	1
19	0	0	1
20	0	1	1
21	0	1	1
23	0	0	1
H1	0	1	1
H2	0	1	0
H3	0	1	1
H4	0	1	0
H5	1	0	1
H6	0	1	0
H7	0	1	0
H8	0	1	0
H9	1	1	0
H10	0	1	0
H11	0	0	1
H12	0	1	0
SBP	0	1	0
CP1	0	0	1
CP2	0	0	0
CP5	0	1	1
CP8	0	1	0
LAC	0	1	0
51651	0	1	1
13565	0	0	0
49775	0	0	0
8096	0	0	1
T336	0	1	0

OPA09			
Isolate	Band 1	Band 2	Band 3
1	1	1	0
2	1	1	0
3	1	1	0
4	1	1	0
5	1	1	0
6	1	1	0
7	1	1	0
9	1	1	0
11	1	1	0
12	1	1	0
13	1	1	0
14	1	1	0
15	1	1	0
16	1	1	0
18	1	1	0
19	1	1	0
20	0	0	0
21	1	0	0
23	1	0	0
H1	0	0	1
H2	0	0	1
H3	0	1	0
H4	0	1	0
H5	0	1	1
H6	0	1	0
H7	1	0	0
H8	0	1	0
H9	0	0	0
H10	0	0	0
H11	1	0	0
H12	0	0	1
SBP	0	1	0
CP1	1	0	0
CP2	0	0	0
CP5	1	0	0
CP8	1	0	0
LAC	0	0	0
51651	0	0	0
13565	0	0	0
49775	1	0	0
8096	0	0	0
T336	0	1	0

RAPD primer groups were run at the same time including thermocycler and same agarose gel. Only the most intense bands were recorded.

Table 7: UPMA Dendrogram raw data input

>SA1	0	0	1	1	0	0	1	1	1	0
>SA2	0	1	1	1	0	0	1	1	1	0
>SA3	0	1	1	0	0	0	0	1	1	0
>SA4	0	1	1	1	0	0	1	1	1	0
>SA5	0	1	1	1	0	0	1	1	1	0
>SA6	0	1	1	1	0	1	1	1	1	0
>SA7	0	1	1	0	0	1	1	1	1	0
>SA9	0	1	1	1	0	1	1	1	1	0
>SA11	0	1	1	1	0	1	1	1	1	0
>SA12	0	1	1	1	0	1	1	1	1	0
>SA13	0	1	1	1	0	0	1	1	1	0
>SA14	0	1	1	1	0	1	1	1	1	0
>SA15	0	1	1	1	0	0	1	1	1	0
>SA16	0	1	1	1	0	0	1	1	1	0
>SA18	0	1	1	1	0	1	1	1	1	0
>SA19	0	1	1	1	0	0	1	1	1	0
>SA20	0	1	1	0	0	1	1	0	0	0
>SA21	0	1	0	1	0	1	1	1	0	0
>SA23	0	1	0	1	0	0	1	1	0	0
>H1	0	0	0	1	0	1	1	0	0	1
>H2	0	0	0	1	0	1	0	0	0	1
>H3	0	1	1	0	0	1	1	0	1	0
>H4	0	1	1	1	0	1	0	0	1	0
>H5	0	0	0	1	1	0	1	0	1	1
>H6	0	0	1	0	0	1	0	0	1	0
>H7	0	0	1	0	0	1	0	1	0	0
>H8	0	0	1	0	0	1	0	0	1	0
>H9	0	1	1	0	1	1	0	0	0	0
>H10	0	0	1	1	0	1	0	0	0	0
>H11	1	0	1	1	0	0	1	1	0	0
>H12	0	0	1	0	0	1	0	0	0	1
>CP1	0	0	1	1	0	0	1	1	0	0
>CP2	0	0	1	0	0	0	0	0	0	0
>CP5	0	0	1	0	0	1	1	1	0	0
>CP8	0	0	1	1	0	1	0	1	0	0
>CPNEG	0	0	1	0	0	1	0	0	0	0
>29213	0	0	1	0	0	1	0	0	1	0

An absorbance reading less than 0.100 OD_{600nm} was determined as weak growth due to susceptibility to the tobramycin. The MIC cut off for tobramycin was determined as 0.100 OD_{600nm} for medium to strong growth. This was detected between 1µg/ml and 0.5µg/ml (Table 9)

Table 9: Tobramycin concentration effect on bacterial cells and OD_{600nm}

Concentration	OD_{600nm}
1µg/ml	0.086
0.5 µg/ml	0.108
0.25 µg/ml	0.114
0.125 µg/ml	0.136
0.0625 µg/ml	0.160

Figure 5: Cut off value was deemed at 0.100

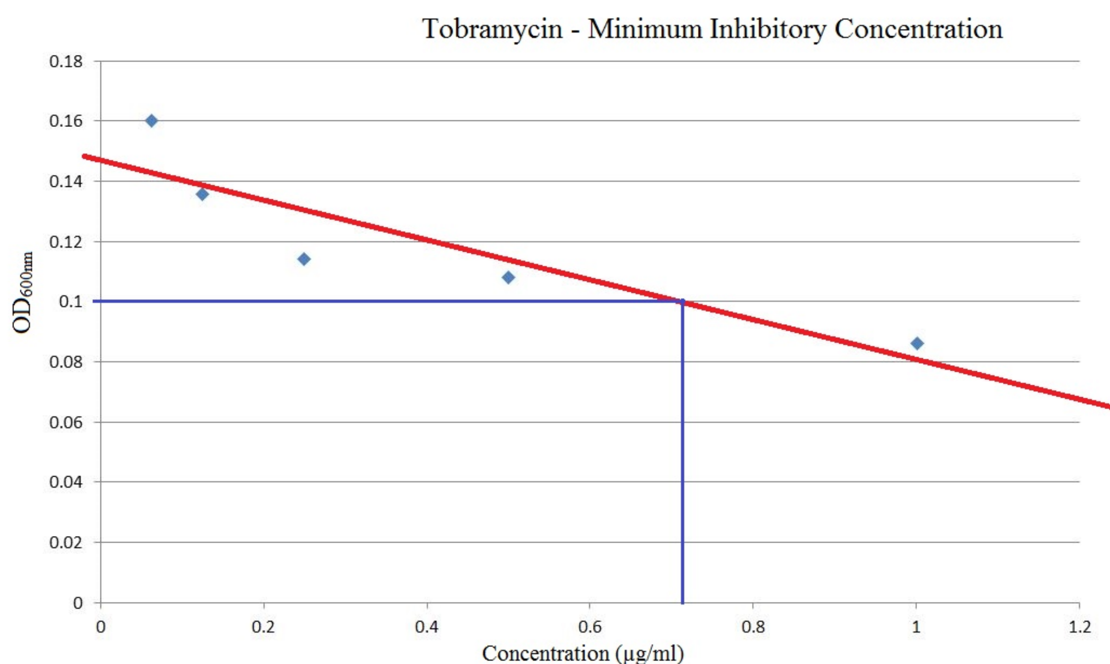
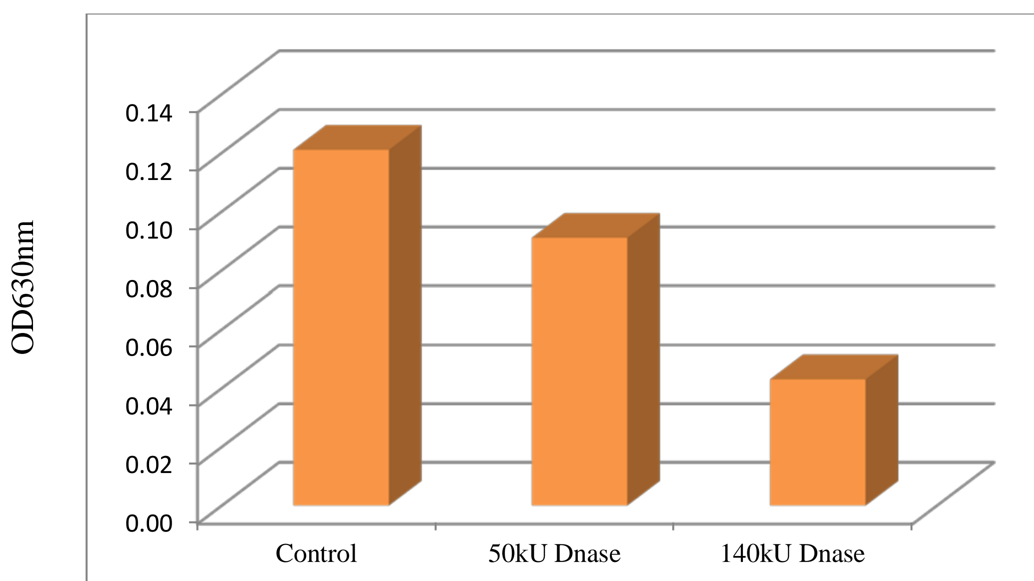


Table 10: Determination of DNase 1 concentration against Biofilm following staining by Crystal violet with standard error mean (±)

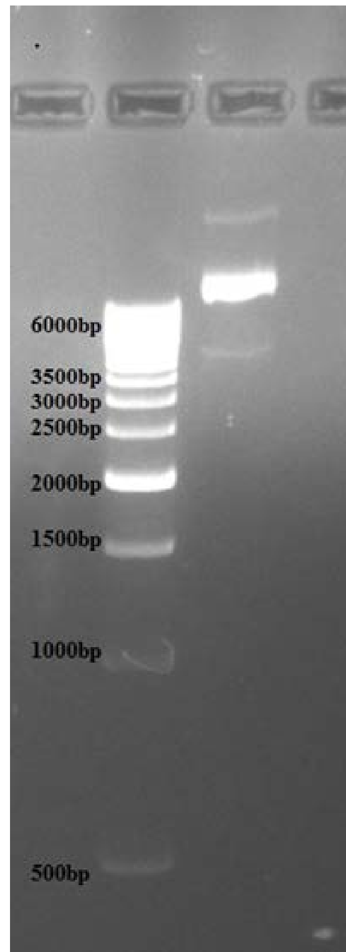
	Control	50kU DNase	140kU DNase
Best 3 values	0.246	0.229	0.178
	0.248	0.212	0.179
	0.247	0.209	0.149
Average minus blank±SEM	0.121±0.001	0.091±0.006	0.043±0.010
P-value	N/A	0.045	0.015

Fig 6: Determination of DNase 1 concentrations with 3 concentration listed in Table 10



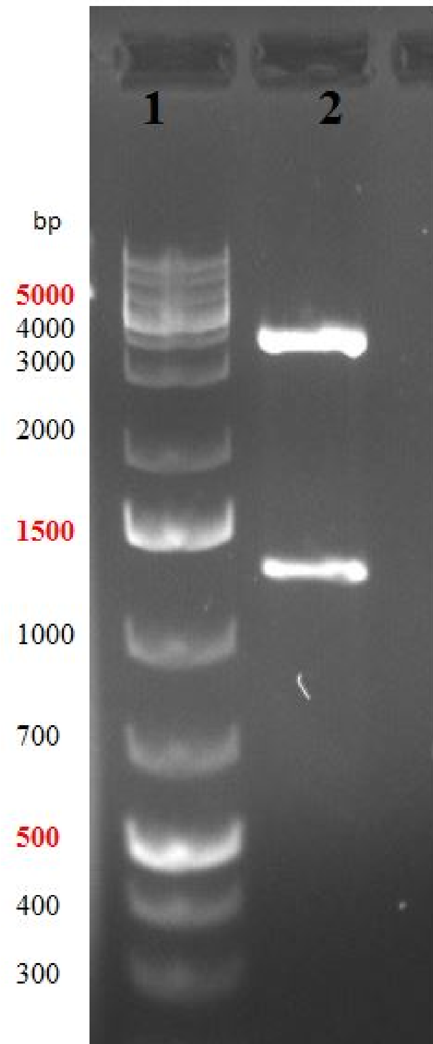
Following administration of DNase and/or control, cells were washed and stained with crystal violet. ODs were obtained at 630nm following solubilisation in 96% Methanol.

Fig 7: Verification of plasmid DNA on 1% agarose gel



pDispersin after purification using the AxyPrep Plasmid MiniPrep Kit from transformed *E. coli*. Lane 1 contains Hyperladder I (Bioline) with bp standards as indicated. Lane 2 contains plasmind DNA. Gel was run with 1% agarose in 1xSB buffer at 90V for 45mins.

Fig 8: Plasmid size verification following restriction enzyme digests.

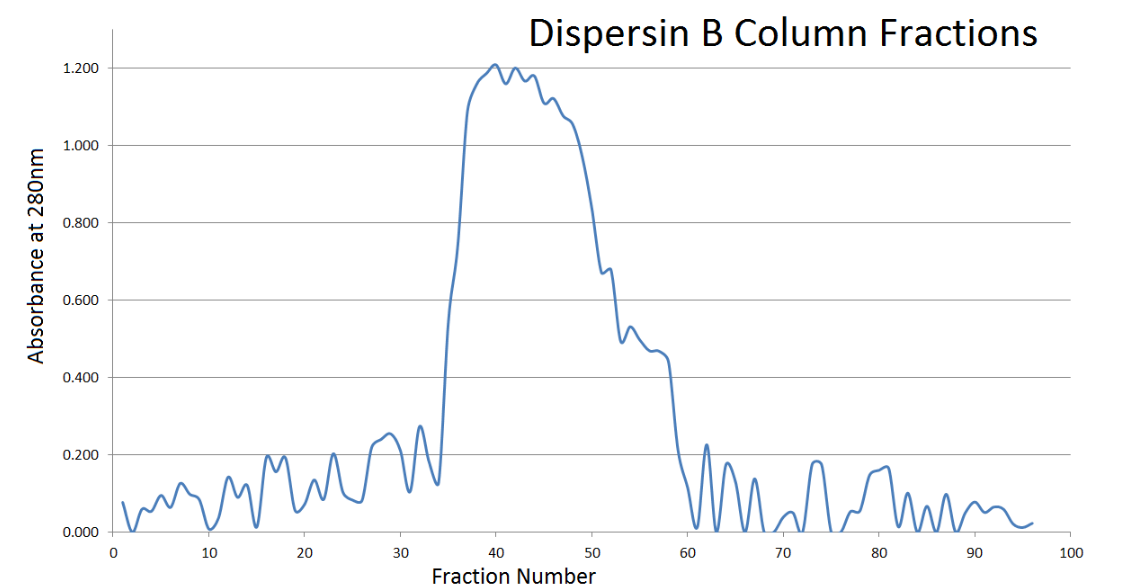


pDispersin was double digested with HindIII and XbaI. Gel was run with 1% agarose in 1xSB buffer at 90V for 1hr. Lane 1 contains the O'Generuler 1kb Plus DNA Ladder (ThermoScientific). Lane 2 contains digested plasmid DNA.

Table 11: Fractions were collected via column chromatography and were recorded with the following absorbance at OD_{280nm}. Fraction numbers 36 to 52 contained dispersin B were pooled and dialyzed overnight.

Fraction No	Abs @ 280nm	Fraction No	Abs @ 280nm	Fraction No	Abs @ 280nm
1	0.077	31	0.104	61	0.012
2	0.000	32	0.273	62	0.226
3	0.059	33	0.181	63	0.000
4	0.054	34	0.129	64	0.174
5	0.095	35	0.539	65	0.130
6	0.064	36	0.743	66	0.000
7	0.126	37	1.087	67	0.138
8	0.098	38	1.159	68	0.000
9	0.084	39	1.186	69	0.000
10	0.008	40	1.208	70	0.039
11	0.036	41	1.159	71	0.050
12	0.142	42	1.200	72	0.000
13	0.090	43	1.166	73	0.175
14	0.121	44	1.179	74	0.174
15	0.013	45	1.109	75	0.000
16	0.193	46	1.121	76	0.000
17	0.156	47	1.076	77	0.053
18	0.192	48	1.054	78	0.055
19	0.056	49	0.970	79	0.147
20	0.072	50	0.836	80	0.160
21	0.135	51	0.671	81	0.165
22	0.085	52	0.678	82	0.014
23	0.203	53	0.494	83	0.101
24	0.103	54	0.531	84	0.000
25	0.083	55	0.497	85	0.067
26	0.082	56	0.469	86	0.000
27	0.219	57	0.468	87	0.098
28	0.240	58	0.441	88	0.000
29	0.254	59	0.211	89	0.050
30	0.211	60	0.115	90	0.078

Fig 9: Elution profile of Dispersin B



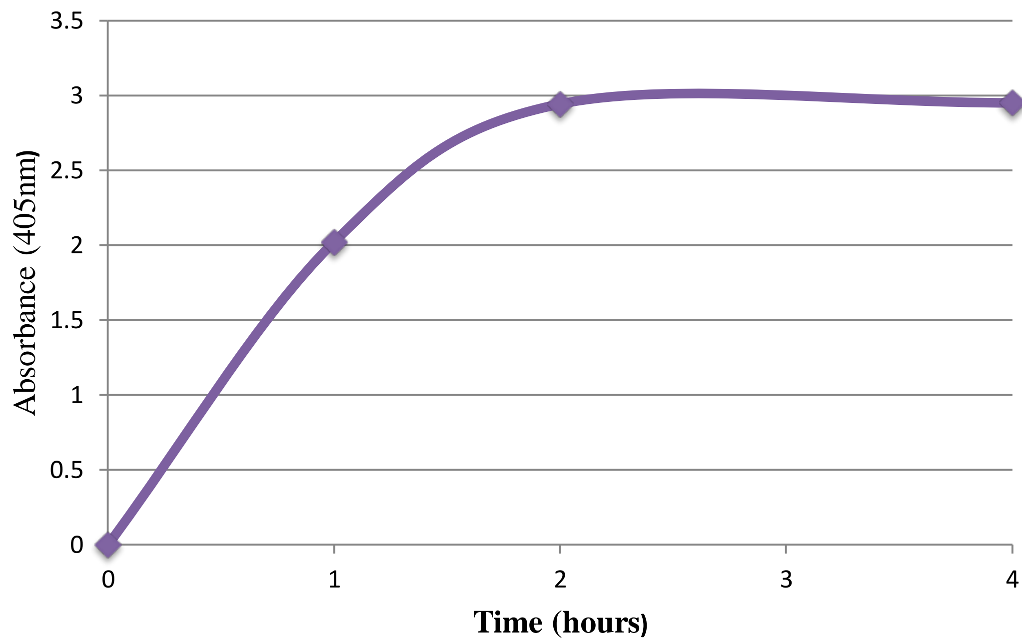
Fractions 36 – 52 representing the enzyme activity pooled

Table 12: Determination of purified dispersin B activity against biofilm ± SEM

Concentration of Dispersin	*Set 1	*Set 2	Average ±SEM	P-value
Neat	1.008	1.190	0.818 ± 0.091	0.02
500µg/mL	1.013	1.213	0.832 ± 0.100	0.03
100µg/mL	1.058	1.239	0.868 ± 0.091	0.02
50µg/mL	1.177	1.181	0.898 ± 0.002	0.05
10µg/mL	1.751	1.780	1.487 ± 0.015	0.14
0µg/mL	1.913	2.031	1.691 ± 0.059	N/A

* Duplicate sets of experiment were performed

Fig 10: Release of p-nitrophenolate from 4-nitrophenyl-N-acetyl-β-D-glucosaminide, by purified dispersin B over a 4-hour timeframe, confirming the presence of glycosyl hydrolase activity.



Chapter 7

Protein identification of fructose-biphosphate adolase class 1

Protein sequence coverage: 27%

Matched peptides shown in ***bold red***.

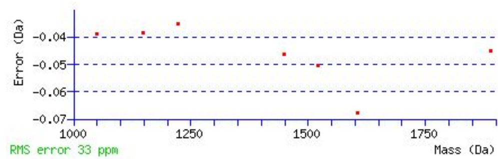
```
  1 MNKEQLEKMK NGKGFIAALD QSGGSTPKAL KEYGVNEDQY SNEDEMFQLV
 51 HDMRTRVVTS PSFSPDKILG AILFEQTMDR EVEGKYTADY LADKGVVPFL
101 KVDKGLAEEQ NGVQLMKPID NLDSLLDRAN ERHIFGTKMR SNILELNEQG
151 IKDVVEQQFK VAKQIIAKGL VPIIEPEVNI NAKDKADIEK VLKAELKKGL
201 DSLNADQLVM LKLTIPTEAN LYKDLAEHPN VVRIVVLSGG YSREKANELL
251 KDNAELIASF SRALASDLRA GQSKEEFDKA LGDAVESIYD ASVNKN
```

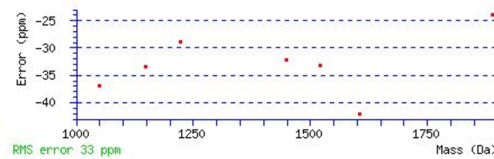
Unformatted sequence string: 296 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start - End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
37	14 - 28	1448.6915	1447.6842	1447.7307	-0.0465	0	99	3.1e-06	1		K.GFIAALDQSGGSTPK.A
40	68 - 80	1522.7428	1521.7355	1521.7861	-0.0506	0	25	73	3		K.ILGAILFEQTMDR.E + Oxidation (M)
45	169 - 183	1605.8534	1604.8461	1604.9137	-0.0676	0	60	0.023	1		K.GLVPIIEPEVNINAK.D
21	224 - 233	1149.5627	1148.5554	1148.5938	-0.0384	0	82	0.00019	1	U	K.DLAEHPNVVR.I
15	234 - 243	1050.5555	1049.5482	1049.5869	-0.0387	0	84	0.00012	1	U	R.IVVLSGGYSR.E
50	246 - 262	1890.9467	1889.9394	1889.9846	-0.0452	1	142	1.2e-10	1	U	K.ANELLKDNAELIASFSR.A
29	252 - 262	1222.5709	1221.5636	1221.5989	-0.0353	0	99	3.4e-06	1	U	K.DNAELIASFSR.A





Data obtained by mass spectrometry performed by Proteomics International the revealed possible identity of a Fructose-biphosphate adolase class 1 from *S. aureus*. Protein sequence coverage was 27%.

Table 13: Immunization schedule and details

Candidate Antigen	Total number for immunization or sham-immunization	Antisera production	Samples collected	Challenge ***	Samples collected* ***
FAB-1*	17 mice	12 mice	Blood	5 mice	Blood, Liver, Spleen
Manganese Transport Protein C	17 mice	12 mice	Blood	5 mice	Blood, Liver, Spleen
Control PBS**	16 mice	11 mice	Blood	5 mice	Blood, Liver, Spleen
Newly identified Fructose-biphosphate aldolase (FAB-1)** ****Phosphate Buffered Saline** ******Challenge 1x10^7 CFU/mL** ***** Weigh organs to enable calculation CFU/g of tissue**					

Table 14: Antibody titers of antisera from mice sham-immunized with PBS (control), aldolase and MntC determined by indirect ELISA

Titers → Group ↓	100	200	400	800	1600	3200	6400
Control	0.014	0.024	0.013	0.013	0.000	0.000	0.000
Aldolase	0.243	0.135	0.087	0.071	0.011	0.000	0.000
MntC	1.504	1.041	0.729	0.496	0.197	0.125	0.070

Table 15: Data analysis of IgM levels between 3 groups ± SEMs

Table - IgM Levels – OD at 405nm ± SEM			
No	Anti-PBS Control	Anti-FBA 1 aby*	Anti-MntC aby
1	0.042 ± 0.002	0.098 ± 0.007	0.043 ± 0.002
2	0.060 ± 0.003	0.127 ± 0.006	0.035 ± 0.003
3	0.041 ± 0.001	0.132 ± 0.006	0.035 ± 0.001
4	0.041 ± 0.003	0.133 ± 0.041	0.043 ± 0.009
5	0.036 ± 0.004	0.065 ± 0.006	0.043 ± 0.001
P-value	N/A	0.0048	0.04986

*aby = antibody

Fig 11: Data analysis showing OD_{405nm}

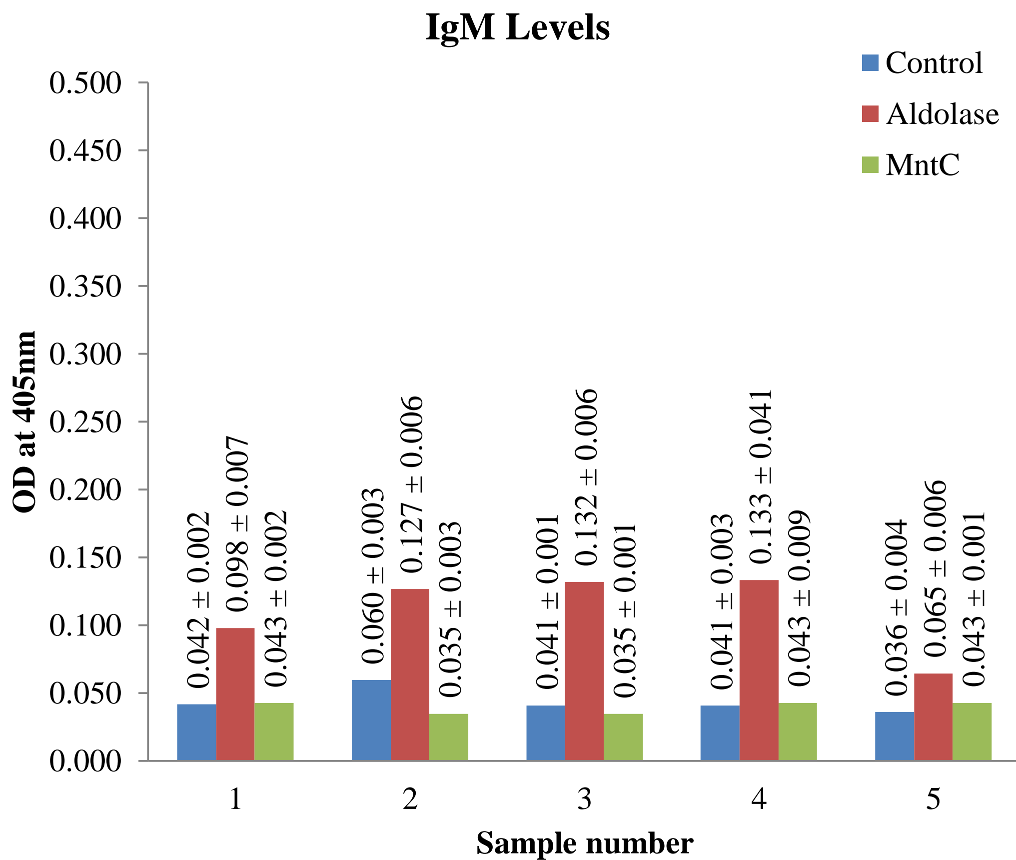


Table 16: Data analysis of IgG1 levels between 3 groups ± standard error means

Table 3 – IgG1 Levels – OD at 405nm ± SEM			
No	Anti-PBS Control	Anti-FBA I aby*	Anti-MntC aby*
1	0.078 ± 0.015	0.087± 0.013	0.807± 0.196
2	0.079± 0.016	0.088± 0.019	0.897± 0.170
3	0.085± 0.015	0.080± 0.013	0.774± 0.258
4	0.082± 0.026	0.091± 0.019	0.816± 0.122
5	0.091± 0.029	0.104± 0.036	1.443± 0.421
P-value	N/A	0.0758	0.0022

*aby = antibody

Fig 12: Data analysis showing OD_{405nm}

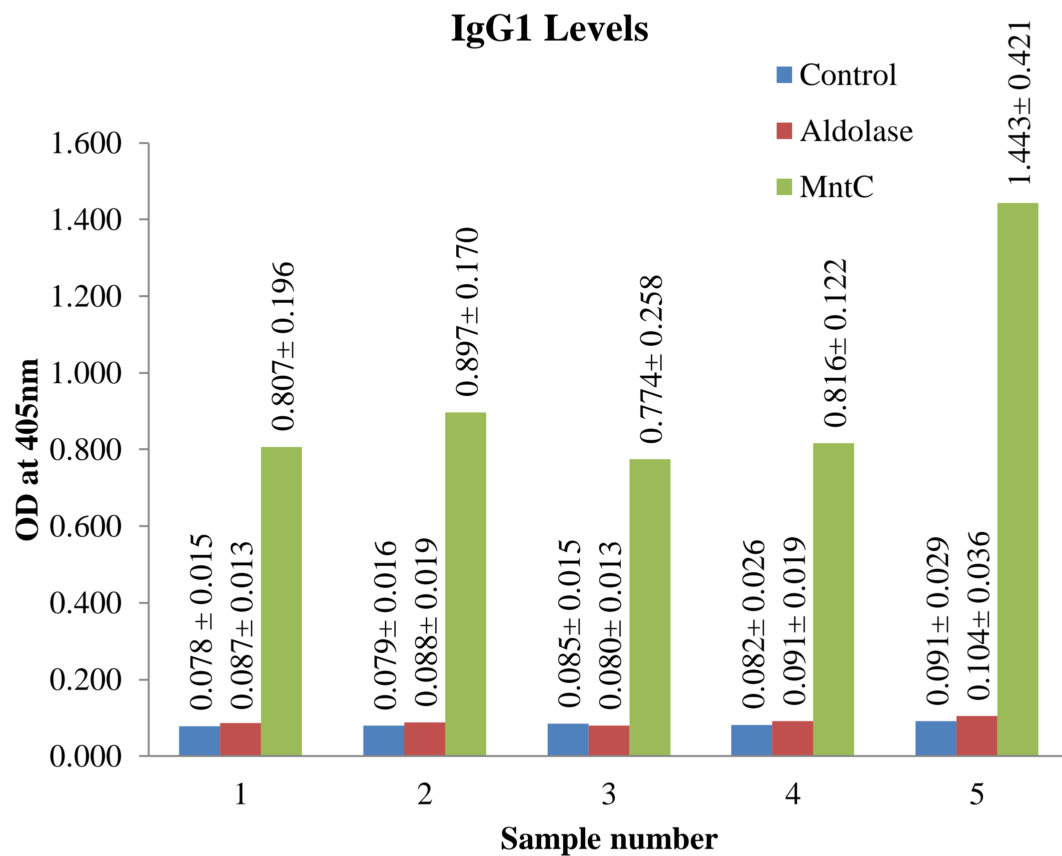


Table 17: Data analysis of IgG2a levels between 3 groups ± standard error means

Table 4 – IgG2a Levels – OD at 405nm ± SE			
No	Anti-PBS Control	Anti-FBA I aby*	Anti-MntC aby*
1	0.073± 0.004	0.036± 0.015	0.134± 0.055
2	0.074± 0.009	0.048± 0.020	0.146± 0.061
3	0.114± 0.049	0.040± 0.018	0.150± 0.050
4	0.056± 0.013	0.039± 0.026	0.152± 0.060
5	0.072± 0.024	0.061± 0.035	0.149± 0.062
P-value	N/A	N/A	0.0023

*aby = antibody

Fig 13: Data analysis showing OD_{405nm}

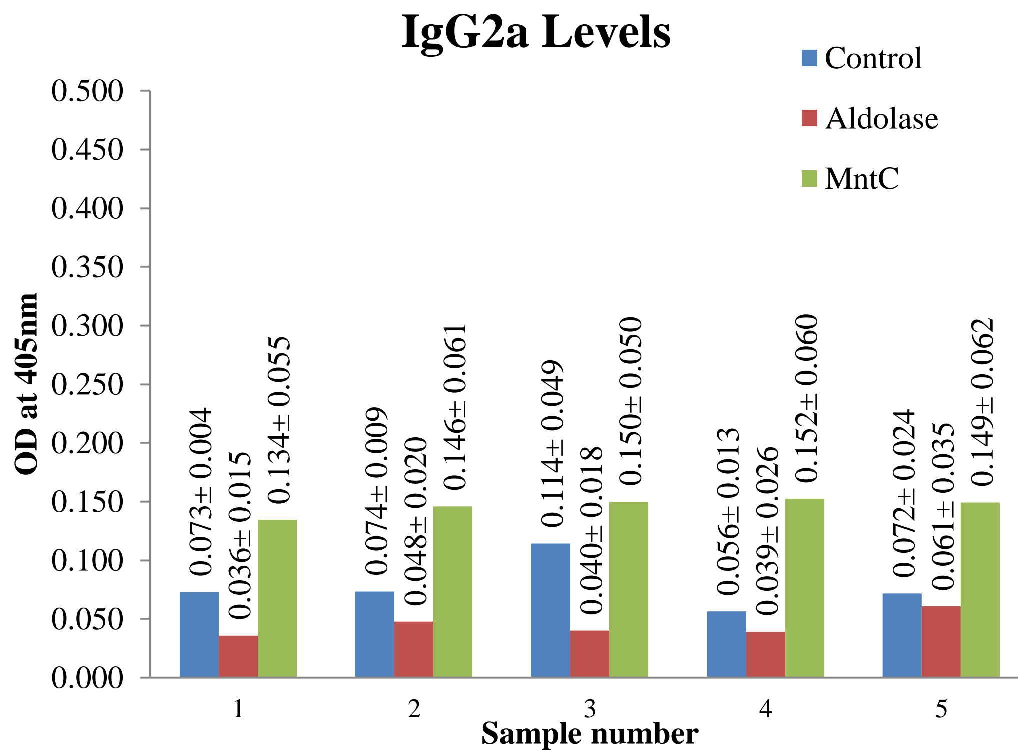
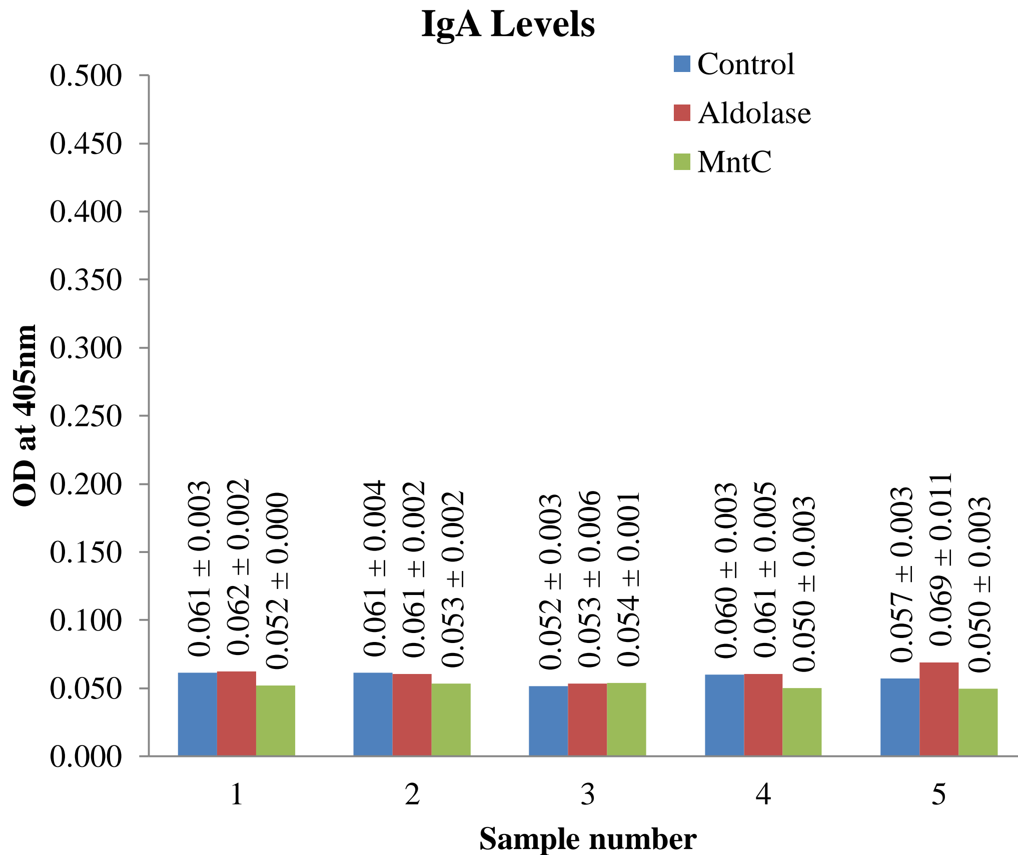


Table 18: Data analysis of IgA levels between 3 groups ± standard error means

Table 5 – IgA Levels – OD at 405nm ± SE			
No	Anti-PBS Control	Anti-FBA I aby*	Anti-MntC aby*
1	0.061 ± 0.003	0.062 ± 0.002	0.052 ± 0.000
2	0.061 ± 0.004	0.061 ± 0.002	0.053 ± 0.002
3	0.052 ± 0.003	0.053 ± 0.006	0.054 ± 0.001
4	0.060 ± 0.003	0.061 ± 0.005	0.050 ± 0.003
5	0.057 ± 0.003	0.069 ± 0.011	0.050 ± 0.003
P-value	N/A	0.2715	0.0450

*aby = antibody

Fig 14: Data analysis showing OD_{405nm}



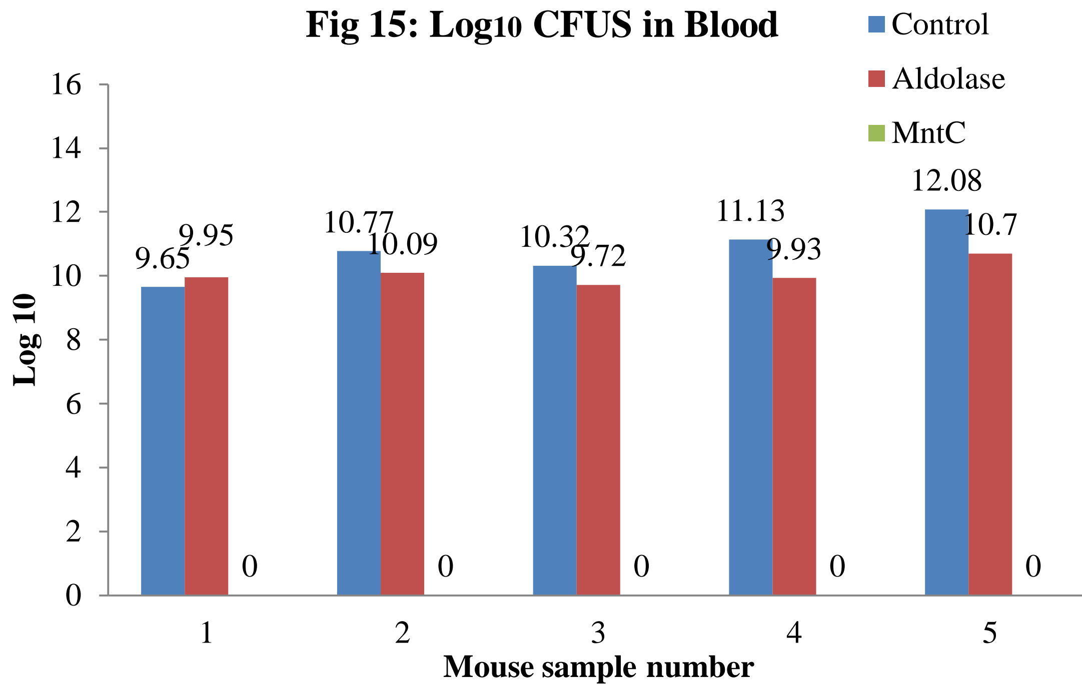
Blood and organ collection were serially diluted till countable colonies were obtained (Tables 19-21). Blood CFUs were calculated at 100µL whereas organ CFUs were caluclated per 100mg.

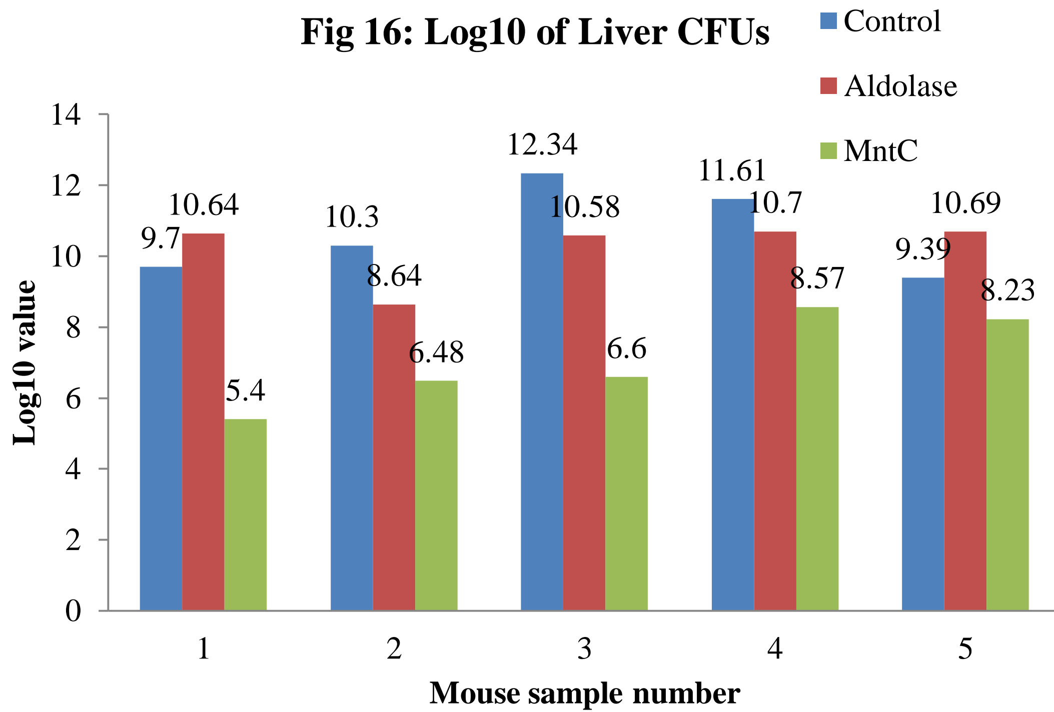
Table 19 – Control group CFUs per mL			
Control No	Blood	Liver	Spleen
1	4.49×10^{9}	5.04×10^{9}	3.36×10^{12}
2	5.85×10^{10}	1.98×10^{10}	2.08×10^{12}
3	2.11×10^{10}	2.17×10^{12}	1.41×10^{12}
4	1.36×10^{11}	4.03×10^{11}	1.63×10^{12}
5	1.19×10^{12}	2.48×10^{9}	3.88×10^{12}
P-value	N/A	N/A	N/A

Table 20 – Aldolase group CFUs per mL			
Aldolase No	Blood	Liver	Spleen
1	8.97×10^{9}	4.39×10^{10}	1.19×10^{10}
2	1.22×10^{10}	4.35×10^{8}	1.84×10^{8}
3	5.19×10^{9}	3.83×10^{10}	4.35×10^{10}
4	8.59×10^{9}	5.07×10^{10}	6.90×10^{7}
5	5.06×10^{10}	4.89×10^{10}	1.14×10^{10}
P-value	0.071	0.312	0.005
Fold Reduction	16.477 fold	14.276 fold	184.604 fold

Table 21 – MntC group CFUs per mL			
MntC No	Blood	Liver	Spleen
1	0	2.53×10^{5}	0
2	0	3.05×10^{6}	0
3	0	3.99×10^{6}	0
4	0	3.75×10^{8}	0
5	0	1.69×10^{8}	0
P-value	0.000	0.008	0.000
Fold Reduction	N/A	4725.946	N/A

Fig 15 Analysis of log_{10} CFU per mL of blood





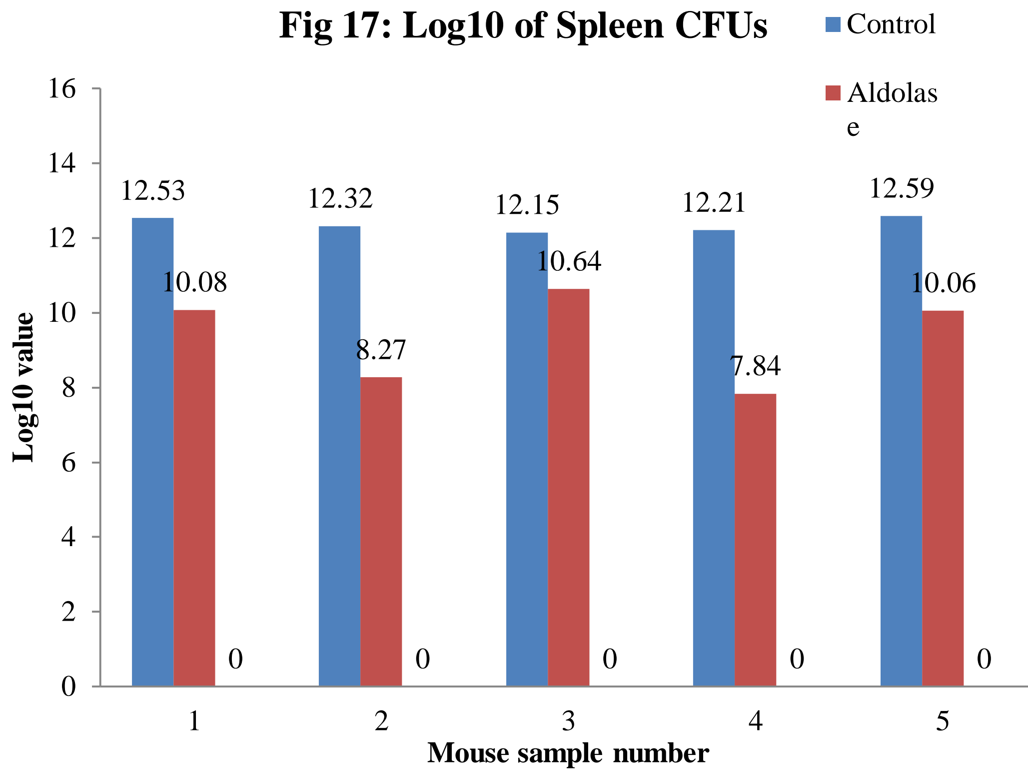


Fig 17: Log10 of Spleen CFUs
Control
Aldolas
e
16
14
12
10
8
6
4
2
0
Log10 value
12.53
10.08
0
12.32
8.27
0
12.15
10.64
0
12.21
7.84
0
12.59
10.06
0
1
2
3
4
5
Mouse sample number

Table 22: Summary of data used in this chapter

Biofilm Inhibition Assay SEM = standard error mean, aby = antibody							
		CFU/mL	Average			CFU/mL	Average
Anti *S. aureus* aby	Set 1	330	380	Control	Set 1	930	1115
	Set 2	430			Set 2	1300	
	Set 3	380			Set 3	1115	
	SEM	28.88			SEM	106.81	
		CFU/mL	Average			CFU/mL	Average
Anti α toxin aby	Set 1	920	1000	Control	Set 1	3860	4370
	Set 2	1040			Set 2	4880	
	Set 3	1040			Set 3	4370	
	SEM	40.00			SEM	294.45	
		CFU/mL	Average			CFU/mL	Average
Anti ClfA aby	Set 1	2020	1920	Control	Set 1	4920	4533
	Set 2	1820			Set 2	4280	
	Set 3	1920			Set 3	4400	
	SEM	57.74			SEM	196.41	
		CFU/mL	Average			CFU/mL	Average
Anti Bbp aby	Set 1	3320	3720	Control	Set 1	4920	4533
	Set 2	3360			Set 2	4280	
	Set 3	4480			Set 3	4400	
	SEM	380.18			SEM	19.64	
		CFU/mL	Average			CFU/mL	Average
Anti isdA aby	Set 1	730	876.67	Control	Set 1	1230	1193
	Set 2	940			Set 2	1120	
	Set 3	960			Set 3	1230	
	SEM	73.56			SEM	36.67	
		CFU/mL	Average			CFU/mL	Average
Anti sdrD aby	Set 1	200	260	Control	Set 1	650	650
	Set 2	200			Set 2	600	
	Set 3	380			Set 3	700	
	SEM	60			SEM	28.87	

		CFU/mL	Average			CFU/mL	Average
Anti isdB aby	Set 1	480	505	Control	Set 1	650	650
	Set 2	530			Set 2	600	
	Set 3	505			Set 3	700	
	SEM	14.44			SEM	28.87	
		CFU/mL	Average			CFU/mL	Average
Anti sdrE aby	Set 1	290	240	Control	Set 1	200	300
	Set 2	190			Set 2	300	
	Set 3	240			Set 3	400	
	SEM	28.87			SEM	57.74	
		CFU/mL	Average			CFU/mL	Average
Anti FnBPa aby	Set 1	100	120	Control	Set 1	200	250
	Set 2	110			Set 2	300	
	Set 3	150			Set 3	250	
	SEM	15.28			SEM	28.87	
		CFU/mL	Average			CFU/mL	Average
Anti SE aby against A,C, D	Set 1	1140	1086.67	Control	Set 1	1280	1196.67
	Set 2	1100			Set 2	920	
	Set 3	1020			Set 3	1390	
	SE	35.28			SE	141.93	
		CFU/mL	Average			CFU/mL	Average
Anti SE aby against A,B, C	Set 1	940	950	Control	Set 1	1280	1335
	Set 2	950			Set 2	1335	
	Set 3	960			Set 3	1390	
	SEM	5.77			SEM	31.75	
		CFU/mL	Average			CFU/mL	Average
Anti Spa aby	Set 1	3000	2960	Control	Set 1	2760	2660
	Set 2	2800			Set 2	2820	
	Set 3	3080			Set 3	2400	
	SEM	83.27			SEM	131.15	
		CFU/mL	Average			CFU/mL	Average
Anti TSST aby	Set 1	3600	3653.33	Control	Set 1	2760	2660
	Set 2	3560			Set 2	2820	
	Set 3	3800			Set 3	2400	
	SEM	74.24			SEM	131.15	

		CFU/mL	Average			CFU/mL	Average
Anti PNAG aby	Set 1	240	235	Control	Set 1	784	778
	Set 2	230			Set 2	780	
	Set 3	235			Set 3	770	
	SEM	2.89			SEM	4.16	
		CFU/mL	Average			CFU/mL	Average
Anti MntC aby	Set 1	1760	1840	Control	Set 1	2520	2680
	Set 2	1760			Set 2	2840	
	Set 3	2000			Set 3	2680	
	SEM	80.00			SEM	92.38	
		CFU/mL	Average			CFU/mL	Average
Anti Aldo-lase aby	Set 1	2400	2040	Control	Set 1	2520	2680
	Set 2	2000			Set 2	2840	
	Set 3	1720			Set 3	2680	
	SEM	197.32			SEM	92.38	

Appendix 2

Reprint of publications

Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Human methicillin-sensitive *Staphylococcus aureus* biofilms: potential associations with antibiotic resistance persistence and surface polysaccharide antigens

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The development of persistent antibiotic resistance by human methicillin-sensitive *Staphylococcus aureus* (MSSA) strains and substantial association with poly-*N*-acetyl glucosamine (PNAG) in biofilms is reported in this investigation. Sixteen of 31 MSSA strains under study were found to have developed resistance to one or more antibiotics, with four strains, two of which did not produce biofilms, showing resistance to cefoxitin, undetectable by *mecA* amplification. Antibiotic resistance displayed by 13/14 biofilm-forming *S. aureus* isolates remained persistent for 4 weeks prior to reverting back to the original antibiotic susceptibility, prompting a suggestion of determining antibiograms for clinical *S. aureus* isolates subcultured from biofilms developed *in vitro* as well as planktonic subcultures prepared from the site of infection. While there was correlation of antibiotic resistance with biofilm formation confirming previous reports, this is the first time that persistence of the biofilm-associated antibiotic resistance by *S. aureus* as planktonic cells is reported. Among the two methods used for assessment of biofilm formation, the tissue culture plate (TCP) method revealed that almost all strains were strong or moderate biofilm producers whereas only 19/31 strains were biofilm producers using the Congo Red agar (CRA) method indicating the superiority of the TCP method in detecting biofilm producers. We also observed no association between biofilm formation and major capsule types. However, substantial, although not absolute, association of biofilm formation with PNAG was observed, warranting continued identification of additional surface-associated polysaccharide and/or protein antigens associated with biofilm formation for development of an effective vaccine against *S. aureus* infections regardless of capsular phenotype.

Keywords: *Staphylococcus aureus* / Biofilm / Antibiotic resistance persistence / Polysaccharide intercellular adhesin / *ica* typing / Capsular phenotype

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Introduction

Staphylococcus aureus is a pathogenic gram-positive bacterium that has emerged as a frequent cause of nosocomial or hospital acquired infections [1]. The pathogen can cause a variety of infections ranging from superficial skin, deep seated skin, wound sepsis, pneumonia, septic arthritis, post-surgical toxic shock syndrome, endocarditis, and osteomyelitis to name a few [1–4]. In a hospital setting, patients who have been surgically treated with indwelling devices or catheters have a higher rate of *S. aureus* infections [5]. There has been an increasing trend in resistance towards β-lactam antibiotics which gives rise to a severe health issue in hospital and community settings [6]. Many nosocomial *S. aureus*

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strains have been shown to be resistant to methicillin (MRSA) [5], spread of which, associated with both nosocomial and community-acquired infections (CA-MRSA), has been reported in all continents [7]. Resistance of this bacterium to antibiotics leads to difficulty in successfully treating invasive and non-invasive *S. aureus* infections. In the United States, high incidence of invasive MRSA infections have been observed, with death in about 20% of all infections, as compared to other pathogenic strains [6], with increasing incidence also observed in the UK and Australia [1, 8].

Persistence of *S. aureus* in infections is dependent on a multiplicity of virulence factors promoting establishment of infection and invasion, and evading the host immune responses [6]. One of the most important virulence factors is the ability of this organism to form biofilms [1]. Biofilm or polysaccharide slime [9] has a major impact on medical implants as it increases bacterial tolerance towards antimicrobial agents and penetration of host defense elements [10]. Importantly, MRSA strains that form biofilms also develop resistance to all the commonly used antibiotics to which the planktonic bacteria are susceptible [1]. The aims of this study were to (a) determine antibiotic susceptibility profile of methicillin-sensitive *S. aureus* (MSSA) strains isolated from biofilms versus planktonic cultures which required selection of a reproducible method for assessment of biofilm formation and (b) determine potential association of biofilm formation by MSSA with the two major surface-associated polysaccharides viz., polysaccharide intercellular adhesin (poly-*N*-acetyl glucosamine [PNAG]) and the predominant capsular types 5 or 8.

Materials and methods

Collection of human *S. aureus* isolates

Nineteen isolates were kindly donated by the Microbiology Section, School of Biomedical Sciences, Curtin University and 12 strains were isolated from undergraduate students studying medical microbiology following approval by Curtin University's Human Ethics Committee (Approval Number SoBS 04/11). All isolates were stored on cryobeads (Blackaby Diagnostics) at −80 °C for further usage.

Biofilm analysis

a. TPC method: This method was adapted from a procedure carried out according to Patterson et al. [11]. The bacterial strains were grown in a 96 well microtitre plate with nutrient broth in 37 °C orbital shaker (80 rpm) for 24 h. The suspensions were adjusted to 10^8 cfu/ml. Two hundred fifty microliter of each suspension was added to a 96 well flat bottom microtitre plate and incubated at 37 °C for 18 h on an orbital shaker after which they were removed from the shaker and left at 37 °C without shaking for the remaining 6 h. After incubation, cells were washed with sterile saline (three times) and fixed in 96% pure ethanol. Wells were then stained with 2% crystal violet and washed three times with sterile distilled water to remove excess stain. Two hundred microliter of 33% glacial acetic acid was then added to each well and absorbance (OD) measured at 600 nm. The average OD of negative control was subtracted from test values. An accredited strong biofilm producer *S. aureus* ATCC29213 was also included in this study. The arbitrary cut off point used for biofilm formation was 0.120 OD_{600nm} according to Christensen et al. [12]. *S. aureus* strains showing $4 \times OD_{600nm}$ at the cut off point (equivalent to an OD of 0.480) or less OD were considered to represent weakly adherent biofilm forming populations, up to $6 \times OD_{600nm}$ at the cut off point (equivalent to 0.720_{600nm}) as moderately adherent biofilm forming populations and values greater than $6 \times OD_{600nm}$ as strongly adherent biofilm forming populations.
b. Congo Red agar method: Congo Red agar plates were made as described elsewhere [13]. Briefly, plates were inoculated and placed in a 37 °C hot room and observed over 72 h for slime production. A positive result was indicated by the production of black colonies. Weak slime producers were indicated by red/pink growth [13]. This experiment was repeated three times to ensure reproducibility. Accredited strong biofilm producer *S. aureus* ATCC29213 was also included in this investigation.

Antibiotic sensitivity/susceptibility testing method

For a comparison between free planktonic and biofilm-associated bacteria, antibiotic sensitivity plates (PathWest) were inoculated using the CDS method [14]. Briefly, bacteria were grown in 2 ml of nutrient broth supplemented with 2% glucose. Broths were left in 35 °C for 48 h to allow adequate biofilm development, after which the supernatant was removed. Bacteria grown in biofilm and free-floating bacteria were streaked for single colonies on MH plates (PathWest). Single colony for each was stabbed with a straight wire, suspended in 2.5 ml saline and flooded onto Sensitest plates (PathWest). Plates were dried for 15 min in 37 °C hot room after which the

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following antibiotics discs (Oxoid) were carefully placed on each plate: benzylpenicillin 0.5 μg (P 0.5), cefoxitin 10 μg (FOX 10), cephalexin 100 μg (CL 100), ciprofloxacin 2.5 μg (CIP 2.5), co-trimoxazole 25 μg (SXT 25), erythromycin 5 μg (E 5), linezolid 10 μg (LZD 10), mupirocin 200 μg (MUP 200), rifampicin 1 μg (RD 1), teicoplanin 15 μg (TEC 15), tetracycline 10 μg (TE 10), and vancomycin 5 μg (VA 5). Zones of inhibition 6 mm or greater were recorded as sensitive except VA5 and TEC15 where zones greater than or equal to 2 mm were recorded as sensitive.

DNA extraction

Using an extraction kit (MO-Bio), all 31 the *S. aureus* strains were placed for DNA extraction. All extracts were stored at −20 °C until required for experimentation after which they were thawed and placed on ice.

Capsular polysaccharide (CP), icaA/D and mecA typing

DNA extracts of the 31 *S. aureus* isolates were subjected to PCR for CP types 5 or 8, *ica* A/D and *mecA* gene expression as follows:

a. Capsular polysaccharide typing: For CP typing, primers published by Moore and Lindsay [2] were used (CP5 forward 5′-ATGACGATGAGGATAGCG-3′ and CP5 reverse 5′-CTCGGATAACACCTGTTGC-3′; and CP8 forward 5′-ATGACGATGAGGATAGCG-3′ and reverse 5′-CACCTAACATAAGGCAAG-3′). Predicted product sizes and Tm were 880 and 1147 bp, and 60 °C and 53 °C, for CP5 and CP8, respectively. PCR cycling condition were 95 °C for 5 min, 95 °C for 30 s, Tm for 30 s, 72 °C for 5 min (×25) and extension at 72 °C for 5 min. PCR product was electrophoresed in 1xTAE buffer in a 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen, Perth, WA).
b. *ica* typing: DNA extracts of the 31 *S. aureus* isolates were run against *ica*A and *ica*D primers published by Vasudevan et al. [15]. The primers used for *ica*A and *ica*D typing were *ica*A forward was 5′-CCTAAC TAACGAAAG GTAG-3′, *ica*A reverse 5′-AAGATA-TAGCGA TAAGTG C-3′; and *icaD* forward 5′-AAACG-TAAGAGAGGTGG-3′ and *ica*D reverse 5′-GGCAATATGATCAAGATAC-3′, respectively. Predicted band size for *ica*A was 1315 bp with a Tm of 48 °C and predicted band for *ica*D was 381 bp with a Tm of 47 °C. PCR run cycle was 95 °C for 5 min, 95 °C for 45 s, Tm for 45 s, 72 °C for 5 min (×30) and extension at 72 °C for 5 min. PCR product was run in 1xTAE buffer in a 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen).
c. *mecA* typing: Detection of the *mec*A gene was carried out as described previously [16] using the following primers: *mec*A forward 5′-AAAATCGATGG-TAAAGGTTGGC-3′ and *mec*A reverse 5′-AGTTCTG-CAGTACCGGATTTGC-3′. Predicted band size was 533 bp with a Tm of 52 °C. The PCR was run on a cycle of 94 °C for 5 min, 95 °C for 30 s, Tm for 30 s, 72 °C for 60 s (×25) and extension at 72 °C for 10 min. The PCR product was then electrophoresed in 1xTAE Buffer on a 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen).

Results

Using the TCP method, 31 strains were assessed for biofilm production including one strong biofilm producing ATCC *S. aureus* strain 29213. This method revealed that all human *S. aureus* isolates were biofilm producers with 14 (45.2%), 15 (48.4%), and 2 (6.4%) strains showing strong, moderate, and weak biofilms, respectively (Table 1). Using the CRA method, colonies that are red or dark red in color indicate negative biofilm production. Colonies that stained black were labeled as biofilm producers. Out of 31 strains including ATCC, 12 (38.7%) samples were positive with black colonies (biofilm producers) whereas 19 (61.3%) were negative for biofilm production with red colonies after 72 h (37 °C). The ATCC *S. aureus* strain 29213, an accredited strong biofilm producer, also displayed dark black colonies as anticipated. It was thus clear that the TCP method was better than the CRA method for detection of biofilm producers despite the observation of varying degrees of biofilm formation (Table 1).

Using the CP typing method, 11/31 strains were CP5 positive (35.5%), 15/31 were CP8 positive (48.4%) with five strains being untypeable (16.1%; Table 1). Twenty-three (23) of the 31 strains used in this study revealed possession of both *ica*A and *ica*D genes, which were either CP5 or CP8 positive. Nine (9) of the 23 *ica*A *ica*D positive strains were moderate biofilm producers whereas 13/23 strains were strong biofilm producers. Out of the remaining nine strains, one strain was positive for either *ica*A with the remaining four strains being positive for the *ica*D gene only. Three *S. aureus* strains that were all CP negative were also *ica*A and *ica*D negative (Table 1).

Antibiotic sensitivity tests on the planktonic cultures of human *S. aureus* isolates revealed that they were all MSSA with similar results being obtained *mec*A gene typing (data not shown). However, when assessed for antibiotic susceptibility of *S. aureus* isolated from the

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Table 1. Typing of human *S. aureus* isolates.

Strain number	CP5	CP8	*icaA*	*icaD*	CRA	TCP
SA 1	−	−	−	+	−	+/−
SA 2	−	+	−	+	−	+
SA 3	+	−	−	+	+	+
SA 4	−	+	+	+	+	+
SA 5	−	−	+	−	+	+
SA 6	+	−	−	+	+	+
SA 7	+	−	+	+	−	+
SA 9	+	−	+	+	+	++
SA 11	−	+	+	+	−	+
SA 12	+	−	+	+	−	+/−
SA 13	−	+	+	+	−	++
SA 14	−	+	+	+	−	+
SA 15	−	+	+	+	−	+/−
SA16	−	+	+	+	−	++
SA 18	+	−	+	+	+	++
SA 19	+	−	+	+	−	++
SA 20	+	−	+	+	−	+
SA 21	+	−	+	+	−	++
SA 23	−	−	+	+	−	+
SA-H1	−	−	−	−	−	+
SA-H2	−	−	−	−	−	+
SA H3	−	+	+	+	+	++
SA H4	−	+	+	+	+	+
SA H5	−	−	−	−	+	++
SA H6	+	−	+	+	−	++
SA H7	+	−	+	+	+	++
SA H8	−	+	+	+	−	+
SA H9	−	+	+	+	+	++
SA H10	−	+	+	+	+	++
SA H11	−	+	+	+	−	++
SA H12	−	+	+	+	−	+
SA ATCC29213	+	−	+	+	+	+

For CP typing, Ica typing, and CRA, results listed as negative (−) and positive (+).
For TCP method, results listed as negative (−), weak (+/−), moderate (+), and strong positive (++).

biofilms, 16 of these isolates had developed resistance towards TE 10, TEC 15, P 0.5, CIP 2.5, SXT 25, CL 100 and FOX 10 upon cultivation as biofilms (Table 2).

It was thus clear that a high rate of resistance to antibiotics developed when MSSA isolates were grown as biofilms. To determine the persistence of antibiotic resistance, *S. aureus* from biofilms were subcultured for 30 days and their antibiotic resistance profile determined at day 30 when it was discovered that the resistance to most antibiotics was maintained (Table 3) as judged by the fact the persistence of antibiotic resistance by 13/14 moderate to strong biofilm forming *S. aureus* strains.

Of the 16 strains that developed antibiotic resistance, 14 strains were either strong or moderate biofilm producers whereas two strains were weak biofilm formers indicating an excellent correlation between antibiotic resistance and biofilm production. On the other hand, 11/31 of human *S. aureus* biofilm producing isolates were encapsulated indicating a lack of correlation of the capsule with biofilm formation. On the other hand, 23/31 MSSA strains that were *ica*A *ica*D positive (74%) were biofilm producers indicating a substantial but not absolute correlation with biofilm formation/ production.

Eleven of the 16 antibiotic resistant strains, 11 strains (68.75%) *S. aureus* possessed both *ica*A and *ica*D genes, essential for production of PNAG [17], a potential contributor to biofilm formation, indicating a substantial relationship with antibiotic resistance. Three of the 16 antibiotic resistant strains were CP negative, seven CP5 positive and six CP8 positive indicating a lack of correlation of antibiotic resistance with encapsulation. One *ica* negative strain (SA-H2) that was classified as a moderate biofilm producer, developed resistance to benzylpenicillin 0.5 μg (P 0.5) and cefoxitin 10 μg (FOX 10). Of the four cefoxitin resistant strains, one was CP negative while the other three were CP5 positive. All four strains were found to have retained their

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Table 2. Development of antibiotic resistance in MSSA strains in biofilms.

	Staphylococcus aureus [SA] strains							
Antibiotic	**SA1**	**SA3**	**SA4**	**SA6**	**SA7**	**SA9**	**SA12**	**SA16**
TE 10	S → R	R	S	R	S → R	S	→R	R
RD 1	R	R	S	R	R	S	R	S
TEC 15	S → R	S	S → R	S	S	S	S	S
FOX 10	S	S → R	S	S	S → R	S	S	S
P 0.5	S → R	S	S	S	R	S	S	R
E 5	S	R	S	S	R	S	S	S
CIP 2.5	S	S	S	S → R	S	S	S	S
SXT 25	S	S	S	S	S	S → R	S	S
MUP 200	S	S	S	S	S	S	S	S
LZD 10	S	S	S	S	S	S	S	S
CL 100	S	S	S	S → R	S → R	S	S	S → R
VA 5	S	S	S	S	S	S	S	S
Strains	**SA19**	**SA23**	**SA-H2**	**SA-H6**	**SA-H8**	**SA-H9**	**SA-H10**	**SA-H11**
TE 10	S → R	R	S	R	S	S	S	S
RD 1	S	S	S	S	S	S	S	S
TEC 15	S	S → R	S	S	S	S	S	S
FOX 10	S	S	S → R	S → R	S	S	S	S
P 0.5	R	R	R	R	R	S → R	S R	S → R
E 5	S	S	S	S	S	S	S	S
CIP 2.5	S	S	S	S	S → R	S	S	S
SXT 25	S	S	R	S	S	S	S	S
MUP 200	S	S	S	S	S	S	S	S
LZD 10	S	S	S	S	S	S	S	S
CL 100	S	S	S	S	S	S	S	S
VA 5	S	S	S	S	S	S	S	S

S → R, sensitive to resistant; S, sensitive; R, resistant.

resistance to cefoxitin after 4 weeks of biofilm formation (Table 3).

Discussion

The resistance of microbial biofilms towards antimicrobial reagents has been the subject of intense interest and yet little is known about the mechanisms of involved. Mah et al. [10] have suggested that maturity of the biofilm is a function of slow growth, stress response and quorum sensing. While biofilms of the common opportunistic pathogens are widely distributed, the resistance mechanisms operating in biofilm formation appear to be distinct from those responsible for conventional antibiotic resistance. However, studies have also shown that

Table 3. Antibiotic resistance profile of *S. aureus* strains after 30 days of subculturing.

	Antibiotic resistance of human *S. aureus* strains	
Antibiotics	**Resistance at day 1**	**Resistance at day 30**
TE 10	SA1, SA3, SA6, SA7, S 12 SA16, SA19, SA23, SAH6	SA1, SA3, SA6, SA7, SA16, SA19, SA23, SAH6
TEC 15	SA1, SA4, SA23	SA1, SA23
P 0.5	SA1, SA7, SA16, SA19, SA23 SAH2, SAH6, SAH8, SAH 9, SAH10, H11	SA1, SA7, SA16, SA19, SA23 SAH2, SAH6, SAH8, SAH 9, SAH10
CIP 2.5	SA6, SAH8	SAH8
SXT 25	SA9	SA9
CL 100	SA6, SA7, SA16	SA7, SA16
FOX 10	SA3, SA7, SAH2, SAH6	SA3, SA7, SAH2, SAH6

SA denotes *S. aureus*.

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biofilm bacteria that were once resistant can revert to sensitivity upon dispersion of the biofilm [18]. The superiority of the TCP method over the CRA method observed in this investigation may indicate potential differences in the surface-associated antigens participating in biofilms formation. The Congo Red dye is a nonspecific dye that has been shown to bind both polysaccharides and proteins [19], nature of which molecules participating in the CRA test has not been elucidated. Our observation on the lack of correlation between the CRA test with biofilm formation by *S. aureus* is supported by a recent report on the lack of correlation of PNAG production with biofilm formation by a gram-negative pathogen, *Yersinia pestis* [20]. However, because of the hydrophilic nature of the surface-associated polysaccharide antigens such as capsule, PNAG and teichoic acids, their potential contribution to biofilm formation in the TCP method may be minimal, unless specifically derivatized, in comparison with that of MSCRAMMs comprising multiple protein structures that are potentially hydrophobic and/or ionic in nature [21, 22].

Formation of biofilm is regulated by a single *icaADBC* operon, which produces the proteins IcaA, IcaD, IcaB, and IcaC [23]. These proteins are involved in the production of the polysaccharide intercellular adhesion, poly-β-1,6-linked *N*-acetylglucosamine or PNAG, the major exopolysaccharide in the *S. aureus* biofilm matrix. The expression of *ica*A and *ica*D genes is of utmost importance in the activation of PNAG synthesis [17]. PNAG is structurally and functionally similar to polysaccharide intercellular adhesion or PIA which is produced by *Staphylococcus epidermidis* [24]. PNAG is considered to be one of the key components of the cell surface that mediates bacterial adherence to host surfaces, enabling biofilm formation and protection [25]. Another component that enables *S. aureus* to resist host defense systems is the production of a capsular polysaccharide or CP. It is generally observed that bacteria that possess an extracellular CP are the "culprit" for invasive diseases [26]. This CP enables the bacteria to evade the host immune response by resisting phagocytosis. The two major serotypes expressed are serotypes 5 and 8 that account for approximately 25–50% of human isolates, respectively [26].

Staphylococci, in particular *S. aureus*, are frequent pathogens in hospital and community acquired settings [1]. This pathogen has emerged as a chronically infecting pathogen, which has demonstrated resistance to multiple antibiotics leading to strains that are methicillin resistant or MRSA [5]. In the US alone, it is estimated that up to 20% of patients undergoing surgery will acquire one or more nosocomial infections costing up to $10 billion [27]. Furthermore, the World Health Organization [28] recently estimated that the overall prevalence of hospital-associated infections in developed countries to be between 5.1% and 11.6%, with (a) more severe a burden in neonatal care, critical care and elderly patients who lack immune function as compared to the general population, and (b) higher rate of mortality in patients who develop septicaemia and pneumonia [28]. The rate of infection in developing countries was found to be several folds higher as compared to developed countries [28].

S. aureus possesses several immune evasion strategies such as production of leukocidal toxins in particular, capsular polysaccharides and Microbial Surface Components Recognizing Adhesive Matrix Molecules or MSCRAMMS [29]. However, one additional strategy of importance for the survival of *S. aureus* is its ability to form biofilms at the site of infection, which renders it resistant to antibiotics. In this investigation, we found that even approximately 50% of *S. aureus* isolates that are MSSA as planktonic cultures acquired resistance to one or more antibiotics upon biofilm formation confirming previous reports [10, 18]. However, we found that the biofilm-associated acquired antibiotic resistance by the *S. aureus* isolates persisted for 4 weeks when grown as planktonic cultures, representing a matter of serious concern in the therapy of staphylococcal infections. While there was an association between biofilm formation and antibiotic resistance developed by MSSA strains, this association was not absolute because of the persistence of the acquired antibiotic resistance as planktonic cells by these strains warranting further investigations. It was interesting, however, that none of the MSSA strains used in this study, that acquired resistance to cefoxitin (4/16) were originally resistant to cefoxitin, the antibiotic used in many pathology laboratories for determination of susceptibility to methicillin [30], with the remaining 12/16 (75%) strains displaying resistance to one or more other antibiotics.

Notwithstanding the suggestion of using more than one method for assessment of biofilm formation of *S. aureus*, the presented data prompts a recommendation that antibiotic susceptibilities of clinical *S. aureus* isolates be determined from cultures of biofilm-associated *S. aureus* developed *in vitro*, in addition to the planktonic cultures prepared directly from the infection site, for optimal therapeutic outcomes particularly for stubborn hospital and community acquired staphylococcal infections including those associated with biomaterial implants [31].

Although there was a general trend of development of antibiotic resistance in *S. aureus* strains expressing both

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*ica*A and *ica*D genes, 75% (12/16) antibiotic resistant strains showing this trait, the correlation was not absolute The absence of 100% correlation of PIA/PNAG of *S. aureus* with biofilm formation is not surprising given the reported participation of other virulence antigens in biofilm formation such as fibronectin-binding proteins, FnBPA and FnBPB [32], collagen-binding adhesion (cna) proteins and clumping factor (clfA) [33].

In summary, our findings suggest that there is no correlation between biofilm formation and encapsulation regardless of the method used for assessing biofilm formation but there is substantial, although not absolute, association with PNAG confirming the need to continue identification and characterization of other polysaccharide and non-polysaccharide MSCRAMMs participating in biofilm formation as is actively being pursued in some laboratories [4, 32, 33]. Our data also suggest that serious consideration should be given to determining antibiograms for *S. aureus* isolated from patients using both biofilms developed *in vitro* as well as planktonic cultures prepared from specimens taken directly from the site of infection for achievement of potentially better therapeutic outcomes.

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Conflict of interest

There is no conflict of interest associated with this study.

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Serological versus molecular typing of surface-associated immune evading polysaccharide antigens-based phenotypes of *Staphylococcus aureus*

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The aim of this study was to compare the performance of serological versus molecular typing methods to detect capsular polysaccharide (CP) and surface-associated polysaccharide antigen 336 phenotypes of *Staphylococcus aureus* isolates. Molecular typing of CP types 1, 5 and 8 was carried out using PCR, whereas serological typing of CP1, 2, 5, 8 and antigen 336 was carried out by slide agglutination using specific antisera. By genotyping, 14/31 strains were CP8 positive, 12/31 strains were CP5 and the remaining 6/31 isolates were non-typable (NT). One isolate was positive for both CP5 and CP8 by PCR, but was confirmed as CP8 type serologically. Detection of CP2 and type 336 by PCR was not possible because specific primers were either not available or non-specific. Using serotyping, 14/31 strains were CP8 positive, 11/31 CP5 positive and 2/31 positive for antigen 336. The remaining four *S. aureus* isolates were serologically NT. However, three of four NT and two 336-positive *S. aureus* isolates were encapsulated as determined by light microscopy after capsular staining. This discovery was surprising and warrants further investigations on the identification and characterization of additional capsular phenotypes prevalent among *S. aureus* clinical isolates. It was concluded that serological typing was a better method than molecular typing for use in epidemiological investigations based upon the distribution of surface-associated polysaccharide antigens-based phenotypes.

INTRODUCTION

Staphylococcus aureus is an important human pathogen causing a broad range of infectious diseases facilitated by its ability to asymptomatically colonize healthy individuals (Daum & Spellberg, 2012; Foster, 2004). The most common conditions associated with this pathogen include wound infections, boils, carbuncles and impetigo, which typically follow abrasions of the skin or mucosal surfaces. The organism can further invade the body or be introduced through medical devices, resulting in systematic infections ranging from osteomyelitis and pneumonia to septicaemia, meningitis and endocarditis (O’Riordan & Lee, 2004; Tzianabos *et al.*, 2001). *S. aureus* is also a common pathogen of immunocompromised patients and a leading nosocomial pathogen in nursing homes, and neonatal care and intensive care units (Ohlsen & Lorenz, 2010).

S. aureus produces several virulence factors, among which the capsular polysaccharides (CPs), which are anti-phagocytic (Sutter *et al.*, 2011), have been widely used as vaccine targets (O’Riordan & Lee, 2004; Robbins *et al.*, 2004). Initial studies, using agglutination tests, reported the existence of

Abbreviations: ATCC, American Type Culture Collection; CP, capsular polysaccharide; NT, non-typable.

11 CP types based on serological specificity (Sompolinsky *et al.*, 1985; Karakawa *et al.*, 1988). However, studies carried out later reported the existence of only four capsular types, 1, 2, 5 and 8, with the remaining types representing mutated forms of one or more of the CP types (O'Riordan & Lee, 2004, Fattom *et al.*, 1998). Many previous studies reported the majority of human *S. aureus* strains (70–80 %) to possess either CP5 and/or CP8 (Skurnik *et al.*, 2010; Roghmann *et al.*, 2005; Verdier *et al.*, 2007), which underpinned the rationale of targeting these two predominant types for the development of conjugate vaccines against infections caused by *S. aureus*. *S. aureus* strains that harboured the capsule locus for CP5 or CP8, but were non-typable (NT) by serological methods for CP1, 2, 5 and 8, were labelled as serotype 336, a surface-associated polysaccharide antigen that is a variant of *S. aureus* cell wall teichoic acid (Sutter *et al.*, 2011). The aim of this study was to compare the performance of serological versus molecular typing methods in determining the distribution of different surface-associated capsular and somatic polysaccharide 336 phenotypes of *S. aureus* isolated from Western Australians.

METHODS

Collection of isolates. A total of 31 *S. aureus* isolates were used in this investigation. A total of 19 of these isolates were obtained from Royal Perth Hospital and Queen Elizabeth II Hospital in Perth, Western Australia, and 12 isolates were collected from undergraduate laboratory medicine students in the School of Biomedical Sciences, Curtin University, Perth, Western Australia (Human Ethics approval no. SoBS 04/11). Positive controls used in this investigation were *S. aureus* strain M (CP1), Smith Diffuse (CP2), strain Newman (CP5), USA 400 (CP8), LAC USA 300 (CP negative) and an antigen 336-positive American Type Culture Collection (ATCC) *S. aureus* strain, 55804.

DNA extraction. Prior to use, the strains were freshly cultured in nutrient broth (PathWest media) with a cryobead, followed by incubation overnight in a shaking incubator at 37 °C. DNA was extracted using a commercial kit (MO-Bio; GeneWorks) and stored at −20 °C. All extracts were thawed on ice prior to genotyping using PCR.

Genotyping of CP types. The PCR primers (GeneWorks) used in this study are shown in Table 1. PCR parameters for *cap1* and *cap2* were as follows: 94 °C for 5 min (initial denaturation); then 25 cycles of 94 °C for 30 s (denaturation), T_m for 30 s (annealing) and 72 °C for 60 s (extension); and 72 °C for 5 min (final extension). PCR parameters for *cap5* and *cap8* were the same as described previously (Babra *et al.*, 2014).

PCR products were separated in a 1.5 % agarose gel in 1 × TAE buffer and the gel was stained with 8 μl Midori Green l^{-1} (Nippon Genetics). The positive controls used for the PCR were strain M (CP1), Smith Diffuse (CP2), strain Newman (CP5) and USA 400 (CP8), and LAC USA 300 was used for the negative control.

CP serotyping. Serotyping was carried out using an agglutination test as described elsewhere (Verdier *et al.*, 2007). CP-specific antisera were raised in specific pathogen-free Quackenbush mice, against CP1, CP2, CP5, CP8 and antigen 336 according to the protocol of J. Gogoi-Tiwari and others (unpublished). Briefly, mice were immunized with *S. aureus* strains M (CP1), Smith Diffuse (CP2), Newman (CP5), USA MW2 (CP8), USA LAC 300 (CP negative) and ATCC 55804 (336) using the following immunization schedule. The first three doses were administered at days 0, 7 and 14. Each dose (0.2 ml per mouse, subcutaneous) consisted of formalin-killed *S. aureus* without an adjuvant (5×10^7, 1×10^8 and 5×10^8 c.f.u. at days 0, 7 and 14, respectively). The fourth and fifth doses contained 1×10^9 and 5×10^9 c.f.u., respectively, mixed equally with the Imject Alum adjuvant (Thermo Scientific). Non-specific reactivity of the typing sera was eliminated by cross-absorption with appropriate *S. aureus* cells of different serotypes, including the accredited antigen 336 strain (ATCC 55804).

Microscopic detection of capsules. The capsules were stained using a modified Maneval's method (Maneval, 1941; Engelkirk & Duben-Engelkirk, 2008). Briefly, the modified method involved scraping of biofilm-associated cells, which were spun down at 6000 r.p.m. for 2 min and the bacterial pellet was washed once with 1 × PBS. Cells were then suspended in a solution of 5 % sucrose and centrifuged at 6000 r.p.m. for 2 min. The supernatant was removed and the pellet was suspended once more in 5 % sucrose. Cells were centrifuged at 9000 r.p.m. for 2 min and the supernatant was removed to obtain as much pellet as possible. Cells in the pellet were gently emulsified in a drop of 1 % Congo red on a clean microscope slide and air dried. The slide was then flooded with Muir's mordant (also known as Maneval's stain) and left to stand for 2 min before rinsing gently with tap water. The slide was then blot dried using clean filter paper and viewed using an oil immersion objective (× 1000 magnification).

RESULTS AND DISCUSSION

A summary of the results obtained using genotyping versus serotyping methods is shown in Table 2. Both genotyping and serotyping methods revealed that none of the strains were positive for CP1. Serotyping was the only effective method for the detection of CP2-positive *S. aureus* strains because the designed primers for *cap2* were non-specific and cross-reacted with the positive control strains for *cap5*, *cap8* and *cap1*, producing 731 bp amplicons (data not shown). However, none of the strains were found to be CP2 positive by serology. Genotyping for *cap5* identified 12/31 strains (38.7 %) to be positive, while one strain produced amplicons of the respective expected sizes for both *cap5* and *cap8* (Table 2). Serologically, however, this strain agglutinated only with anti-CP8 serum. Both the genotyping and serotyping results were in agreement for CP8, where 14/31 (45.16 %) of the isolates were positive. As primers for type 336 were not available, genotyping for antigen 336 could not be carried out at this time. Using PCR, 6/31 (19.35 %) of the isolates were regarded as NT isolates. A total of 2 of the 6 NT strains, or 2/31 (6.45 %) of the total isolates that were NT either by genotyping or by serotyping, were found to be antigen 336 positive by serotyping. Taken together, a total of 4/31 strains or 12.9 % were regarded as being NT.

All of the strains were then subjected to capsular staining. The bacterial cell stained red/purple against a dark background with the capsules appearing as unstained white haloes. Strain USA LAC 300 (CP negative) and one of our test isolates, H7, which was positive for CP5 by genotyping (Babra *et al.*, 2014) and serotyping (this study),

Table 1. PCR primers used for CP typing

Target gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	T_m (°C)	Expected size (bp)	Reference
cap1	AGG TCT GCT AAT TAG TGC AA	GAA CCC AGT ACA GGT ATC ACC A	57	550	J. Gogoi-Tiwari and others, unpublished
cap2	AGC AAT CTT CGG TTA TTG CCG GTG	ATG ACG GTA AGG CAT CAA GGT CG	60	731	J. Gogoi-Tiwari and others, unpublished
cap5	ATG ACG ATG AGG ATA GCG	CTC GGA TAA CAC CTG TTG C	54	881	Babra *et al.*, (2013)
cap8	ATG ACG ATG AGG ATA GCG	CAC CTA ACA TAA GGC AAG	52	1148	Babra *et al.*, (2013)

were used as negative and positive controls, respectively (Figs 1 and 2). All of the *cap8*-positive isolates were found to have a capsule, as did all the *cap5*-positive isolates, including one strain that was positive for both CP5 and CP8 by PCR but was CP8 positive by serology. Quite surprisingly, it was discovered that three of the four NT isolates were also encapsulated when subjected to capsular staining (Fig. 3).

S. aureus is the cause of multiple disease syndromes in both community and hospital settings. A well-known and established key factor in its virulence is the production of a capsule (Engelkirk & Duben-Engelkirk, 2008), an important immune evasion molecule of *S. aureus* (Nanra *et al.*, 2012). As such it has been used as a target for vaccine development and evaluated as a key component of conjugate vaccines in preclinical models, as well as in human trials (Nanra *et al.*, 2012; Pozzi *et al.*, 2012).

Our study has shown that capsular phenotypes 5 and 8 were the predominant capsular phenotypes among the Western Australian *S. aureus* isolates included in this investigation. However, we found that serological typing using slide agglutination was better for determining capsular phenotype than the genotyping method because of the lack of availability of specific primers for detection of CP2 and antigen 336. Serologically, 80.6 % of the total *S. aureus* isolates were composed of CP8 (45.16 %) and CP5 (35.48 %), confirming previous reports from select other countries (Roghmann *et al.*, 2005; Verdier *et al.*, 2007;

Table 2. Summary of genotyping and serotyping results

Capsular type	No. (and percentage) of isolates
Detection by PCR	
CP1	0 (0 %)
CP2	NA*
CP5	12 (38.7 %)†
CP8	14 (45.16 %)
336	PNA
NT	6 (19.35 %)
Detection by serology	
CP1	0 (0 %)
CP2	0 (0 %)
CP5	11 (35.5 %)
CP8	14 (45.16 %)
336	2 (6.45 %)
NT	4 (12.9 %)
Detection by staining	
Positive reference strains M (CP1), Smith Diffuse (CP2), Newman (CP5) and MW2 (CP8)	Capsule visible on all the strains
Negative reference strain US LAC 300	No capsule visible
CP5 and CP8 seropositive isolates	Capsule visible on 26 out of 26
NT	No capsule visible on 1 out of 4 Capsule visible on 3 out of 4
336 positive	Capsule visible on 2 out of 2

NA, Not applicable; PNA, primers not available.

*The primers produced non-specific bands with positive controls for *cap1*, *cap5* and *cap8*, as well as bands for *cap2*.

†Includes one strain that showed positive for both CP5 and CP8 by genotyping; however, it was confirmed to be CP8 by serotyping.

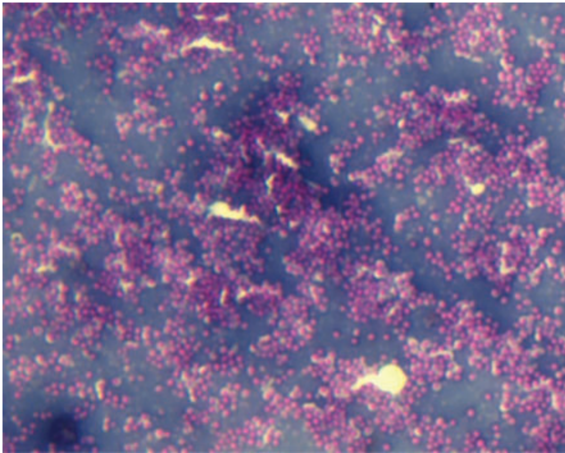

Fig. 1. Negative control *S. aureus* isolate (USA LAC 300) stained by modified Maneval's capsule staining method (×1000 magnification).

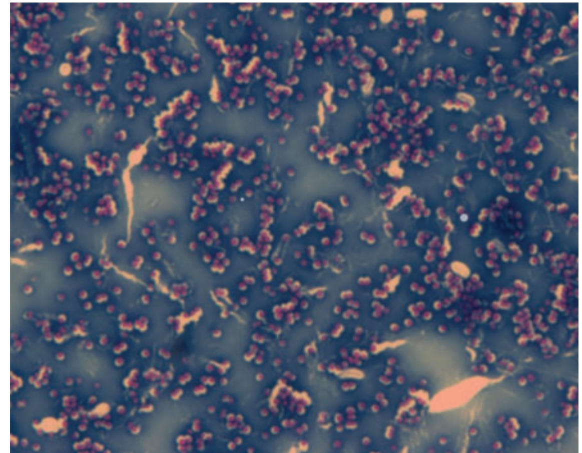

Fig. 3. NT CP isolate of *S. aureus* (CP negative by genotyping and serotyping) displays a capsule using modified Maneval's capsule staining method (×1000 magnification).

Skurnik *et al.*, 2010), the remaining isolates being either antigen 336 positive or NT.

Sompolinsky *et al.* (1985) performed capsular typing of *S. aureus* isolated from human infections for the 11 capsular serotypes by precipitation and agglutination with specific antisera. This research group reported that 63 % of their isolates were type 8, 16 % were type 5, 2 % were type 7 and 0.3 % were type 10, with more than 90 % of total isolates being encapsulated. The remaining 10 % of the encapsulated isolates were not identified as belonging to the 11 known capsular types, in these isolates the capsule may represent a prototype capsule that is different from the accepted 11 serotypes (Sompolinsky *et al.*, 1985). This is in contrast to a previous report that the lack of expression of a capsule by NT strains was due to random point mutations

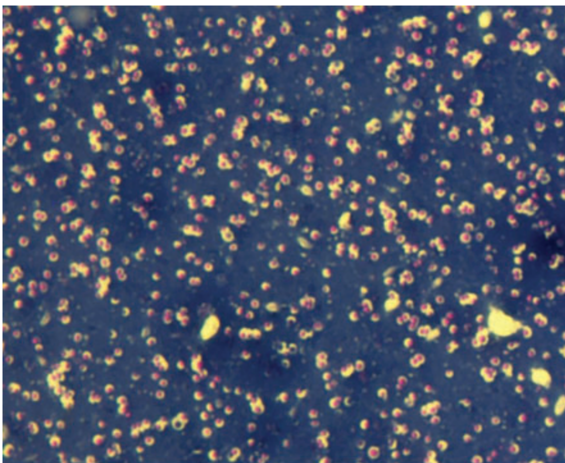

Fig. 2. Positive CP control *S. aureus* isolate (H7) stained by modified Maneval's capsule staining method (×1000 magnification).

in the CP5A promoter or replacement by the insertion sequence IS*257* (Cocchiaro *et al.*, 2006). Our study demonstrated the existence of more serotypes than just the four capsular types (CP1, 2, 5, 8) and also raises a question on the validity of the antigen 336 as a somatic non-capsular antigen.

Given that most vaccines have employed surface-associated polysaccharide antigens, particularly CP5 and CP8, conjugated with one or more potential protein adhesins such as alpha toxin, ClfB and IsdB (Pozzi *et al.*, 2012), coupled with the fact that no protection is expected to be imparted against infections caused by NT *S. aureus*, it is important to gain knowledge on the nature of the antigens unique to NT isolates, including new capsular antigens/phenotypes, for the formulation of an improved vaccine against *S. aureus*. Our study has highlighted the potential importance of determining the prevalence of not only the major capsular serotypes, CP5 and CP8, of *S. aureus*, but also other antigens particularly antigen 336. The fact that 75 % of the NT *S. aureus* strains and the antigen 336-positive strain were also found to be encapsulated, even by light microscopy, warrants further investigations on the identification of additional capsular types present among the NT isolates for complete epidemiological investigations and formulation of appropriate conjugate vaccines.

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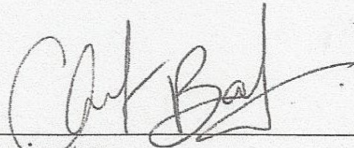
Appendix 3

Written statement of co-authors

To Whom It May Concern,

I, **Charlene Babra Waryah**, as a first author declare myself as the primary contributor to the experimental design, interpretation and writing to the paper/publication entitled **Human methicillin-sensitive *Staphylococcus aureus* biofilms: potential associations with antibiotic resistance persistence and surface polysaccharide antigens.**



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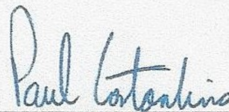
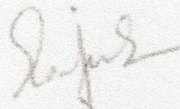


Paul Costantino
Co-author 2



Raju Sunagar
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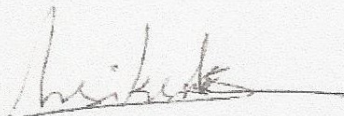

Shrikrishna Isloor
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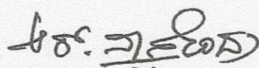

Nagendra Hegde
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Trilochan Mukkur
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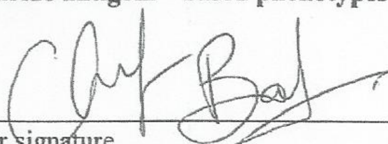

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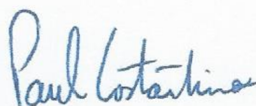

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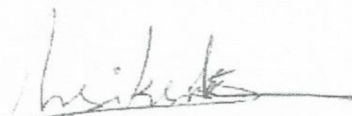

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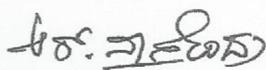

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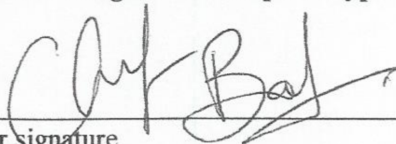

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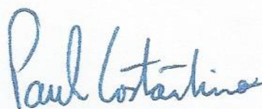

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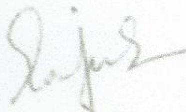

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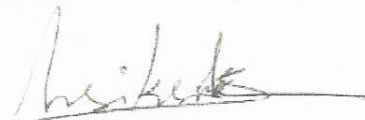

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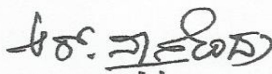

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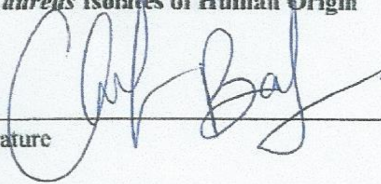

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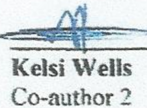

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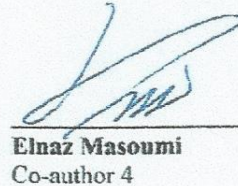

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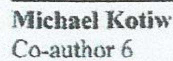

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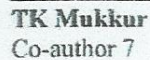

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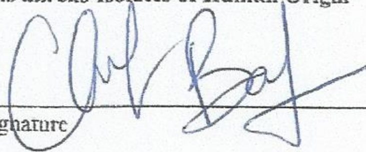

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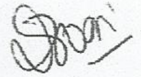

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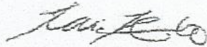

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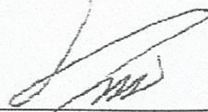

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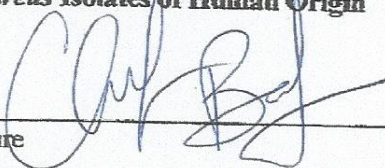

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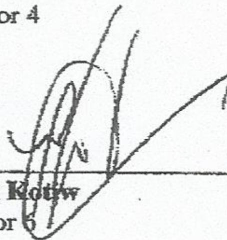

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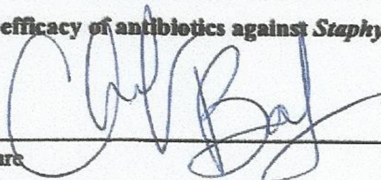

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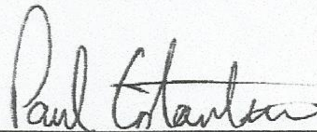

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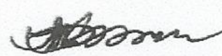

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TK Mukkur
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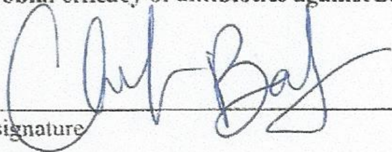

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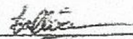

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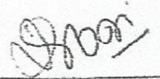

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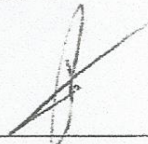

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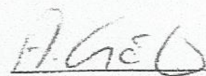

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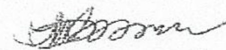

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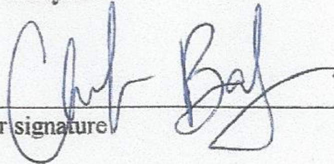

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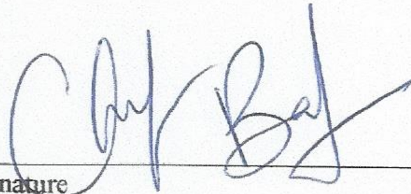

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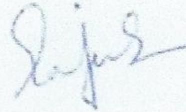

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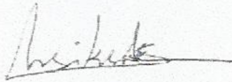

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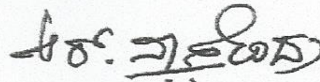

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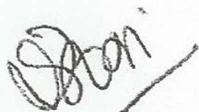

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