Using Phage Display Technology to Produce Peptides Specific for *Staphylococcus aureus* Type 5 and Type 8.

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Abstract.

S. aureus is an important human and animal pathogen which is responsible for a variety of community and hospital acquired infections. *S. aureus* causes 20,000 deaths annually in the United States which exceeds the annual deaths caused by diseases such as HIV/AIDS, influenza and viral hepatitis together. Treatments of infections caused by this bacterium are difficult due to antibiotic resistance and failure of the active and passive immunity to confer the required immunity against this pathogen. Short synthetic peptides play a major role as bioprobes for the detection of microorganisms and biological molecules. Our goal is to produce a specific phage clone to S. aureus type 5 or type 8 using phage display technology. Phage clones displaying peptides specific for *S. aureus* were selected in previous studies. However, high non-specific background was seen in M13 ELISAs when using *S. aureus* type 8 as the ligand. In this study we reduced the high background by changing the blocking buffer from BSA to nonfat dry milk and by repeating trypsinization of the ligand used in the ELISA assays. However, our negative controls were still high, indicating continued non-specific binding of antibody to the plate. To investigate specific binding in the absence of whole cells, we purified capsular polysaccharide from *S. aureus* type 5 using digestion of bacteria paste with proteinase K, DNAse, RNAse and treatment with sodium periodate. The presence of carbohydrate in the column fractions was determined using a red tetrazolium assay. Purity of the carbohydrate was determined by a Bradford protein assay, a phosphate test to detect teichoic acid and light absorbance to detect nucleic acid. The purified carbohydrate was used as the ligand in an ELISA assay. Phage clones NLT8.7 and MMT5.2 were tested for binding. Neither NLT8.7 nor MMT5.2 showed significant binding to the carbohydrate. In future studies we hope to repeat the ELISA assays with the new block and trypsin treated ligand, and test all the phage clones previously selected.

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List of abbreviations.

BSA- Bovine serum albumin

CA- community- acquired.

CA-MRSA- Community- associated methicillin resistant *Staphylococcus aureus* infections.

CDC- Centre for Disease Control and Prevention.

CP5- Capsule polysaccharide type 5.

CP8- Capsule polysaccharide type 8.

DEAE- Diathylaminoethyl cellulose.

ELISA- Enzyme-linked immunosorbent assay.

HA-MRSA- Health care-associated methicillin resistant *Staphylococcus aureus* infections.

HCA- health- care assaociated.

HCl- Hydrochloric acid.

H2SO4- Sulfuric acid.

LA-MRSA- Livestock methicillin resistant S. aureus infections.

MMT5.2- Martin Maratani *S. aureus* type 5 clone two.

MRSA- Methicilin-resistant *S.aureus.*

NAG- N- acetylmuramic.

NAM - N-acetylglucosamine.

NLT8.7- Nina Lenkey S. aureus type 8 clone seven.

OD- optical density

PAMPS- Pathogen associate molecular patterns.

PEG- Polyethylene glycol

PBP- Penicillin- binding proteins.

PRR- pattern recognition receptors.

PVL- Panton- Valentine leucodin.

SAB- S. aureus bacteremia.

S. aureus- Staphylococcus aureus

SpA- *S. aureus* protein A.

SSTI- soft tissue infections.

SSCmec- Staphylococcus chromosome cassettes.

TMB- 3’3’, 5’5-Tetramethylbenzidine.

VISA- Vancomycin intermediate *S. aureus.*

VRSA- Vancomycin resistant *S.aureus.*

CHAPTER 1.

1.1 INTRODUCTION:

1.1.1 Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a commensal and opportunistic pathogen that causes a broad-spectrum of infections which range from minor superficial skin lesions to very severe and sometimes deadly diseases. *S. aureus* invades and infects man and other homoeothermic species. In man *S. aureus* causes skin and soft tissue infections (SSTI), bacteremia, endocarditis, toxic shock syndrome and sepsis. *S. aureus* can be attributed to approximately 20,000 deaths per year which exceeds the deaths caused by diseases such as influenza and HIV/AIDS. *S. aureus* infections are also known to recur in approximately 13% of individuals, which leads to bacteremia in individuals with surgical and antibiotic therapy (Falugi *et al.,* 2013, Smith *et al.,* 2013, Stranger-jones *et al.,* 2006). Antibiotics such as methicillin have been used to treat these infections. Some strains of *S. aureus* are resistant to antibiotics. Monoclonal antibodies used to treat infections have resulted to immune responses in human such as allergies. Vaccines, such as the whole-cell and purified subunits have all failed to reach the human test stage. In these studies phage display technology will be used to obtain peptides that are specific for *S. aureus* type 5 or type 8. These peptides could be coupled to a toxin and used to produce breakthrough in *S. aureus* infections treatment.

The family Staphylococcaceae contains four genera. Of these, the genus *Staphylococcus* is the most important. It consists of small Gram-positive cocci which

range from 0.5-1.5 mm in diameter. These bacteria form large, irregular, three-dimensional clusters like clumps of grapes (Prescott *et al.,* 2005). This structure is observed under a microscope following a Gram stain, where they appear to be bluish or purplish clusters of grapes. They have a cell wall that contains peptidoglycan and teichoic acid. They do not move, nor form spores and they facultative anaerobes that grow by anaerobic respiration and fermentation (Harris *et al.,* 2002).

Micro-organisms in this genus are classified into two groups; pathogenic and non- pathogenic. This classification tends to be based on the ability to synthesize the enzyme coagulase. The coagulase produced by these bacteria causes blood plasma to clot, which protects the bacteria from phagocytosis by white blood cells. *S. aureus* produces a yellow carotenoid pigment, and thus is usually referred as a 'golden staph'. The organism can be detected by its growth characteristics and the beta-hemolysis pattern on a blood agar plate (Prescott *et al.,* 2005).

S. aureus is the most studied of the genus *Staphylococcus* due to its potential to cause a wide range of infections. It is a commensal, which is an organism living within another organism deriving benefits like food and shelter, but usually not harming the host individual (Burgey et al., 2016). It is also an opportunistic pathogen, being able to cause disease when the host resistance has been lowered (Smith *et al.,* 2013). In humans, this pathogen can manifest as a mild benign skin lesion to a very devastating infection (Ragle et al.,2009).

1.1.2 *S. aureus colonization*

S. aureus can cause disease in the host or remain asymptomatic. It can develop in the host with no signs and symptoms. This scenario is known as colonization (Cavalcanta *et al.,* 2005). *S. aureus* is a naturally occurring bacterium in approximately 30% of individuals (Giersing *et al.,* 2016), it appears normally on the skin and nasopharynx of the human body (Harris *et al.,* 2002). The primary reservoir sites include the nasal vestibule which harbors up to 35% of bacteria; perineal region has 20%, the umbilical, axillary and the interdigital regions 5%-10% of the *S. aureus* found in the body (Cavalcanta *et al.,* 2005).

S. aureus causes infections in various parts of the body such as the skin, nose, urethra, vagina and the gastrointestinal tract (Harris *et al.,* 2002). It is estimated that approximately 20-30% of people are colonized with *S. aureus*. The colonization of this bacterium in the host does not harm the host, and the host is asymptomatic, however the continuous presence of this bacterium in the host may lead to an increased risk of contracting an infection. The bacterium can be transmitted to others directly or indirectly by both non-symptomatic and infected persons (Smith *et al.,* 2013).

S. aureus is known to be the leading cause of mortality and morbidity in both hospital acquired, and community acquired infections. Bassett and coworkers (Bassett et al., 2011) conducted research in two Italian University hospitals to determine the epidemiological properties and risk factors that make a person susceptible to health-care associated (HCA) and community-acquired (CA) *S. aureus* bacteremia (SAB). They

identified three case groups. Group one consisted of patients with HCA SAB > 48 hours, group 2 consisted of patients with HCA SAB < 48 hours, and group 3 included the CA SAB. The controls were patients with no *S. aureus* positive clinical culture from any site. They recorded the deaths within 30 days of the first blood culture then compared the survivors and non-survivors to determine the in-hospital mortality caused by SAB. They found that the incidence rate of *S. aureus* infections was 38 per 100,000 patient-days, with 111,455 hospitalized patients in the study. Six patients were excluded from the study due to improper records. Of the 165 left, 112 (67.8%) were classified as HCA SAB >48 hours, 29 (17.5%) were HCA SAB < 48 hours and only 24 (14.5%) were CA SAB. Out of 165 patients 35 (21.2%) died within 30 days after SAB onset (Bassetti *et al.,* 2011). Klein and coworkers (Klein et al., 2007) suggested that hospital acquired infections are estimated to cause >90,000 deaths in the United states only, and that *S. aureus* infections are sixth highest cause of mortality nationally (Klein et al,2007).

1.1.3 Staphylococcus aureus infections.

The ability of *S. aureus* to cause disease is due to combined effect of extracellular factors and toxins, plus the invasive characteristics of the strain. *S. aureus* infections begin by the formation of a localized abscess. When this bacterium becomes established in a hair follicle, the result is a necrotic tissue infection (Prescott *et al.,* 2005).

Coagulase secreted by the bacterium causes the blood to clot forming a fibrin wall around the infected region. In the center of the affected region the necrotic tissue becomes liquid in form; the abscess then spreads to other parts of less resistance. The necrotic tissue at the center then drains and the healing process is initiated.

Unfortunately, in some scenarios the bacteria may spread to other parts of the body through the lymph or the blood stream (Prescott *et al.,* 2005).

This bacterium is highly adaptable, and it's a major cause of lower respiratory tract infections, surgical site infections and nosocomial diseases. Major diseases caused include: septic arthritis, pneumonia, endovascular infections, osteomyelitis, septicemia, boils, abscesses, wound infections and toxic shock syndrome (Giersing *et al.,* 2016, Prescott *et al.,* 2005). *S. aureus* is also a major cause of food poisoning. This was first seen in March, 1986, when there was an outbreak of an acute gastrointestinal illness when 855 people were served supper at a buffet in the New Mexico Country Club. Of these, 67 people became ill with symptoms including diarrhea and vomiting and 24 required urgent hospitalization. The root of this problem was *S. aureus* which was growing in a turkey with dressing that was cooled for 3 hours. During this time it produced toxins that were found in the food when it was served at the buffet (Prescott *et al.,* 2005). The United States Centers for Disease Control and Prevention (CDC) reported that approximately 1 in 120 Americans suffer from food poisoning resulting from *S. aureus* contamination annually (Liu *et al.,* 2016).

1.1.4 The structure of *S. aureus*.

1.1.4.1 Cell wall.

Understanding the cell wall structure *S. aureus* is important to gain a deeper knowledge of the bacterial physiology, how the bacterium interacts with the host to initiate an infection and to understand the mechanism employed by antibiotics to kill or

suppress the bacterium (Umeda *et al.,* 1987). The cell wall of this bacterium is made of 50% peptidoglycan which plays a key role in protection of the bacterium, due to its ability to resist high osmotic pressure and lysis of the cell. Teichoic acids form around 40% of the cell wall weight. Teichoic acid includes both cell membrane-associated lipoteichoic acid and cell wall teichoic acid. These give the bacterium a negative charge, hence playing a significant role in localization of metal ions and activation of autolytic enzymes (Harris *et al.,* 2002). The cell wall also has a layer of proteins, which aid in the interaction of peptidoglycan with the environment. Some proteins are non-covalently bound to peptidoglycan, teichoic acids or other receptors. Those that are covalently bound to peptidoglycan aid in adhesion to host tissue prevent opsonization and block phagocytosis (Prescott *et al.,* 2005).

1.1.4.2 Capsular polysaccharide

The *S. aureus* capsule consists of high-molecular weight polysaccharides (CPS), which are firmly attached to the cell surface. The bacteria use the capsule as a defense mechanism and protective layer which aids in adapting to changes in the environment (Chan *et al.,* 2014). Approximately 90% of *S. aureus* capsular polysaccharides belong to 11 different serotypes. The major serotypes known to be found in hospital acquired infections are the capsule polysaccharides type 5 (CP5) and type 8 (CP8) (Thakker *et al.,* 1998). *S. aureus* CP5 and CP8 share the same repeating units of three sugar residues (one N-acetyl mannosuronic acid and two N-acetyl fucosamines), but differ in the glycosidic linkages and acetylation (Jones, 2005).

Human clinical isolates are 70-80% serotype 5 or serotype 8. Poutrel and coworkers used an enzyme-linked immunosorbent assay to investigate which capsular polysaccharides were expressed by *S. aureus* in isolates from rabbits, poultry, pigs and horses (Poutrel and Sutra 1993). Their findings showed that approximately 98% of the samples were typeable and that 75.8% of poultry and 66.7% of pigs expressed *S. aureus* capsular polysaccharide type 5. *S. aureus* capsular polysaccharide type 8 was expressed in 59.5% of rabbits and 83.3% of horses (Poutrel and Sutra 1993).

Capsular polysaccharide type 5 (Reynolds strain) and capsular polysaccharide type 8 (Becker strain) act as virulence factors that protect against killing of the bacterium through as phagocytosis (Nanra *et al.,* 2013, Nelles *et al.,* 1985, Thakker *et al.,* 1998). The capsule prevents phagocytosis by preventing the binding of pathogen associated molecular patterns (PAMPS). Phagocytes express on their surfaces pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPS). These trigger phagocytosis of the bound microbe. (Reviewed in Owen *et al.,* 2013).

Phagocytosis is the process by which phagocytes (macrophages and the neutrophils) take in particles or bacterium by engulfment. It involves a bacterium attaching to the membranes forming what is known as pseudopodia. A phagosome is formed once the bacterium is ingested. The phagosome then fuses with the lysosomes and lysosomal enzymes lyse and digest the bacterium. The products of digestions are then released from the cell. Phagocytosis can also be indirect where by a phagocyte recognizes a soluble protein that is bound on the microbial surface a process termed as

opsonization. These soluble proteins are known as opsonins. Opsonins are substances that promote phagocytosis of the pathogen by binding to them. An example of an opsonin is the complement fragments produced by the activation of the complement in serum (Reviewed in Owen *et al.,* 2013).

The capsule of the *S. aureus* plays a vital role in the protection against the effects of the complement such as the complement- mediated phagocytosis (Lee *et al.,* 2004). The complement system is the first line of defense against pathogens such as bacteria, viruses, protozoans, helminths and fungi (Lee *et al.,* 2004). Complement itself is a group of serum proteins that are activated by infection, tissue injury and antibodies products of the adaptive immune response. There are three complement activation paths; classical, lectin and the alternative pathways (Reviewed in Owen *et al.,* 2013, Lee *et al.,* 2004). The initiators of the three pathways are different, but at one point they all form C3 convertase. This complex enzyme can cleave the C3 into the C3a and C3b. C3b acts as an important opsonin in bacteria, but its functional is inhibited by the capsule. *S. aureus* can activate all the 3 complement pathways; the lectin, classical and the alternative paths. The anti-phagocytic activity in the encapsulated *S. aureus* was due to the capsule blocking the C3b protein fragments and the diminished deposition of the C3 on the surface of *S. aureus* (Lee *et al.,* 2004).

Thakker and coworkers (1998), investigated the virulence factor of the encapsulated and non- encapsulated *S. aureus* strains. They compared the mouse virulence of *S. aureus* type 5 with the mutant strain (capsule-defective), which were cultivated under high or low capsule expression conditions. They found that,

encapsulated *S. aureus* strain was anti-phagocytic and more virulent compared to the non- encapsulated. The virulence factor in the encapsulated *S. aureus* was as a result of the bacterium resisting killing by human polymorphonuclear leukocytes in the serum (Thakker *et al.,* 1998).

1.1.4.3 Protein A.

Protein A, also abbreviated as SpA, is found in the Staphylococcal bacterial cell wall and is released by the bacteria during its growth (Thammavongsa *et al.,* 2014, Falugi *et al.,* 2013). It acts as a virulence factor that is believed to inhibit opsonophagocytic killing by neutrophils (Nanra et al., 2013). It binds to the Fcγ domain of immunoglobulin. It is this binding ability that enables *S. aureus* to avoid opsonophagocytic killing (Thammavongsa *et al.,* 2014).

Protein A also binds to the Fab domain of immunoglobulin heavy chains, specifically the Fab region of human and mouse variable region of the heavy chain in the V_H3 family of antibodies (Falugi *et al.,* 2013). Cross-linking of the immunoglobulin by protein A occurs, which triggers proliferation of the VH3 clones resulting in expansion and apoptosis of the activated B cells. This results in the prevention of the development of the adaptive immune response (Thammavongsa *et al.,* 2014, Falugi *et al.,* 2013).

1.1.5 Prevention and treatment.

1.1.5.1 Proper hygiene.

To prevent infection with *S. aureus*, proper hand washing should be used at all times when dealing with reservoirs of *S. aureus* bacteria, such as furniture, clothes and

hospital equipment and while working around colonized or infected patients. This is to prevent cross-contamination from infected persons to non-infected persons (Breves *et al.,* 2015). When infections do occur, antibiotics are usually the first line of treatment.

1.1.5.2 Antimicrobial antibiotics.

1.1.5.2.1 Penicillin.

Fleming was the first person to coin the word Penicillin in 1929. He observed that when a *Staphylococcus* strain was streaked on a culture plate and the plate left open on the lab bench, 'molds' grew, and they lysed the bacterial colonies. These molds were identified as Penicillium. To ascertain his observation, he grew Penicillium in broth at room temperature and he confirmed that it had both bactericidal and bacteriolytic activity against Gram positive bacteria, including *S. aureus,* but no observed effect on Gram negative bacteria (Fleming 1929).

Penicillin is known as a beta-lactam antibiotic that was clinically introduced the mid- 1940s. Antibiotics in the penicillin family have a beta-lactam ring that is important in disrupting synthesis of the bacteria cell wall. With the increased frequency of *Staphylococcus* resulting in approximately 80% mortality during the pre-antibiotic era, penicillin became the most effective and reliable antibiotic, since it significantly reduced the infections caused by *S. aureus* (Silvanna *et al.,* 2005). In 1942, reports indicated that *S. aureus* was resistant to beta-lactam antimicrobial agents (Silvanna *et al.,* 2005). Many bacteria produce beta-lactamase, an enzyme that breaks the beta-lactam ring resulting in inactivation of the antibiotic. These enzymes are produced by bacteria and are useful

in providing multi-drug resistance to beta-lactam antibiotics by breaking the antibiotic structure. With continued use of penicillin, alarming reports confirmed that the resistant *S. aureus* infections that had previously been confined to the hospital setting had become more widespread causing infections in the community at large. Unfortunately, by the late 1960s, more than 80% of hospital acquired and community acquired *S. aureus* infections became resistant to penicillin (Klein *et al.,* 2007). This resistance was due to the synthesis of the lactamase by the bacterium, hence it became ineffective (Silvanna *et al.,* 2005).

1.1.5.2.2 Methicillin.

With the increased resistance to penicillin (Penicillin G), scientists began developing a penicillin derivative that could not be hydrolyzed by the beta-lactamase (Silvanna *et al.,* 2005, Smith and Jarvis 1999). The antibiotic was synthesized by substituting the phenol group of the benzylpenicillin with a methoxy group (Silvanna *et al.,* 2005). This new drug was called methicillin.

In 1961 methicilin-resistant *S. aureus* (MRSA) was first detected (Smith et al., 2013, Breves *et al.,* 2015). The resistance was as a result of the synthesis of new penicillin binding proteins (PBPs). PBPs are transpeptidases and carboxypeptidases that participate in synthesis of the bacterial cell wall (Silvanna *et al.,* 2005). *Staphylococcus* is known to produce four penicillin-binding proteins, PBP1, PBP2, PBP3 and PBP4, while MRSA also has another sub-type, PBP2a. PBP2a is encoded by the *mecA* gene. It has a low affinity for the beta-lactam antibiotics which prevent bacterial cell wall assembly.

Hence, it renders MRSA strains resistant to multiple beta-lactam antibiotics (Breves *et al.,* 2015, Silvanna *et al.,* 2005).

S. aureus infections are very difficult to treat. This is due to the gradual change of resistance to antimicrobial drugs. Klein et al., suggests that methicillin resistant *S. aureus* (MRSA) in several regions of the US hospitals, long- term care facilities and communities is already being endemic. Unfortunately, to be more specific, it is epidemic (Klein *et al.,* 2007).

MRSA nasal colonization is seen in approximately 1-2% of the population in the United States alone. Veterans' affairs (VA) hospital patients can have up to 13% nasal colonization and up to 10-15% of patients in acute care and intensive care units are colonized with MRSA (Eko *et al.,* 2015). The National Health and Nutrition Examination Survey found the prevalence of the nasal carriage of *S. aureus* (MRSA) in the community to be 8% of healthcare associated infections. The carriage rate for all *S. aureus* was 32.4%, the carriage of MRSA was associated with age (over 60 years) and gender where females were the most affected. They also found two Staphylococcus chromosome cassettes (SCCmec) (a mobile genetic element) in MRSA. SCCmec type II which was found in the elderly age group and SCCmec type IV that was found in younger age groups. SCCmec was unlikely to encode multiple antibiotic resistance but had a likelihood of carrying a virulence factor called Panton-Valentine leucocidin (PVL), PVL is a pore- forming toxin associated with the formation of necrotic lesion (Reviewed in Cooke and Brown, 2010).

Methicillin-resistant *S. aureus* infections can be transmitted by direct contact from carriers to non- carriers (Giersing *et al.,* 2016). In a hospital setting health workers who are taking care of the patients and handling objects that may be colonized with *S. aureus* may contaminate their hands and ultimately infect other patients (Cavalcanti *et al.,* 2004). These infections are known as health care-associated methicillin resistant *Staphylococcus aureus* infections (HA-MRSA) (Smith *et al.,* 2013).

Community-associated MRSA infections (CA-MRSA) are infections in non-hospitalized persons and healthy persons who have never contracted an illness. A MRSA-positive specimen that is obtained from non-hospital settings or within 48 hours of being admitted to the hospital, or from a person who had not been to hospital within 2 years before the date of MRSA isolation. MRSA has also been reported to be increasing in these individuals (Smith *et al.,* 2013). (Salmenlinna *et al.,* 2002, Smith *et al.,* 2013). CA-MRSA was first detected in the mid- 1990s. It was known to come from a specific group of patients, like military recruits, sports teams and individuals involved in activities that resulted in the skin abrasions. (Cooke and Brown 2010). Two clones of the CA-MRSA were reported in the USA; USA300 and USA400, both harbor the Panton-Valentine leucocidin (PVL) toxin gene. The presence of the PVL resulted in prolonged hospital stays and a larger nosocomial reservoir of CA-MRSA which causes infections within 3 days after hospital admission. (Cooke and Brown 2010).

The advent of livestock methicillin resistant *S. aureus* (LA-MRSA) infections was first observed in Netherlands in 2004, where swine herds were the reservoirs of *S. aureus* in several regions of Europeans countries and North America. In an experiment

by Smith in 2013 (Smith *et al.,* 2013), a screening test was conducted to determine the prevalence of the infection in animals in the European countries. The results showed no LA-MRSA in England, Ireland or Sweden. Animals were 41% positive for LA-MRSA in Germany and 51% positive in Spain. This has raised great concerns about the association between public health and the livestock industries. It has been shown that the ST 398 clonal complex strain which is a livestock-associated methicillin-resistant *S. aureus* is detected in the nasal swabs of pigs, cattle and, in livestock workers (Smith *et al.,* 2013).

Environmental transmission or air transmission is uncommon (Cavalcanti *et al.,* 2005), except for burn units and Intensive Care Units (ICUs), where transmission is seen in tracheostomized patients with pneumonia caused by *S. aureus.* In the ICUs, the prevalence rate was found to be 37.1% (Cavalcanti *et al.,* 2005).

1.1.5.2.3 Vancomycin.

Vancomycin, which become available in 1958, is a glycopeptide antibiotic that is selective for Gram positive bacteria. This antibiotic prevents cell wall formation by complexing with the D-alanyl-D-alanine portion of a phosphodisaccharide-pentapeptide-ligand complex. It is also known to inhibit the incorporation of N-acetylglucosamine (NAM) and N-acetylmuramic acid (NAG) into the growing peptidoglycan chain (Saha *et al.,* 2008).

With increased resistance to beta-lactam antibiotics, including methicillin, and the emergence to a greater extent of community-associated MRSA, this situation was

alarming. As a result, Vancomycin became the drug of choice for the treatment of the *S. aureus* infections in both CA-MRSA and HA-MRSA. (Giersing *et al.,* 2016). Unfortunately, continued use of this antibiotic encouraged bacterial resistance. This was due to gradual changes that occurred in the bacteria. In 1996, the first MRSA with intermediate resistance vancomycin was reported in Japan (Breves *et al.,* 2015). Since this time there have been increased cases of vancomycin intermediate *S. aureus* (VISA) and vancomycin resistant *S. aureus* (VRSA) (Giersing *et al.,* 2016).

1.1.5.3 Active and passive immune mediated therapy.

The excessive use of antibiotics intensified the danger of *S. aureus* contamination, resulting in very serious infections, which are lethal if proper medical care is not available (Liu *et al.,* 2016). The fact that almost all MRSA became resistant to multiple antibiotics, such as penicillin, cephalosporins, chloromycetin, lincomycin, aminoglycosides, tetracycline, and macrolides, including vancomycin (Nian *et al.,* 2016), *S. aureus* thus remains an omnipresent pathogen worldwide (Liu *et.,al* 2016). The spread of antibiotic resistance in communities and hospitals has made therapeutic intervention very difficult and expensive. There is an urgent need to develop active and passive immune mediated therapy that could prevent or treat *S. aureus* infections (Nian *et al.,* 2016).

1.1.5.3.1 Vaccines.

Vaccines are known to prevent infectious diseases that are a threat to human lives by providing active immunity. A vaccine is defined as a preparation immunogenic material that is used to induce immunity against pathogenic organisms (Reviewed in Owen *et al.,* 2013). The aim is to design a vaccine candidate that will maximize the activation of both cellular (T cells) and humoral (B cells) immune responses. Vaccines are classified as live, attenuated when a microorganism is disabled to lose its pathogenicity but can grow in the inoculated host. This attenuation is often achieved by growing the bacteria for an extended period under abnormal culture conditions. The aim is to select mutants that can grow best in this unsuitable condition rather than in the natural host. A second type of vaccine is inactivated or 'killed'. These vaccines are treated with heat or chemicals, killing the pathogen and rendering it unable to replicate, but still capable of inducing an immune response. A third type is subunit vaccines. These contain specific, purified macromolecules that are derived from the pathogen. Two less common, newer vaccines include recombinant vector vaccines and DNA vaccines. Recombinant vector vaccines are made by using the live, attenuated vaccines with added genes encoding antigens that are present in a new pathogen. DNA vaccines utilize plasmid DNA which encodes antigenic proteins and ingested directly into muscles (Reviewed in Owen *et al.,* 2013).

Live vaccines stimulate a very strong immune response with a long-lived immunity, but may mutate into virulent form. Killed vaccines on the other hand are stable, safer than live vaccines but they stimulate a weaker immune response (Reviewed

in Owen *et al.,* 2013). This may pose a danger to patients if they have been unable to generate the required protective immunity (Stranger-Jones *et al.,* 2006). Sub-unit vaccines can contain inactivated exotoxin, capsular polysaccharide or key recombinant protein antigens. These vaccines have been the best choice to confer protective immunity in pneumococcal pneumonia, a disease caused by *Streptococcus pneumonia*. The vaccine induces the formation of opsonizing antibodies (Reviewed in Owen *et al.,* 2013).

Sub-unit vaccines for *S. aureus* infections have been developed which are composed of individual surface proteins, such as clumping factor A (ClfA), clumping factor B (ClfB), iron-regulated surface determinant B (IsdB) or fibronectin-binding proteins (FnBP). These can generate partial protection against *S. aureus* infection (Reviewed in Owen *et al.,* 2013, Yukiko *et al.,* 2006). The vaccine protection is less reliable, however in cases of infection after vaccination, the infection will be less severe than in an unvaccinated individual.

Polysaccharide vaccines containing capsule have been attempted for several diseases. Polysaccharides are unable to activate the T helper cell. Polysaccharide sub-unit vaccines activate B cells through a thymus-independent type 2 manner without T cell help. As a result, IgM is produced with very little class switch, no affinity maturation and little or no development of memory cells (Reviewed in Owen *et al.,* 2013).

S. aureus protein A (SpA), has been used to produce a sub-unit vaccine. As described previously, SpA binds to the constant region of antibody, preventing it from

opsonizing the bacterium and preventing phagocytosis. SpA also cross-links antibodies on the surface of B cells, causing abnormal B cell function. Faluga and coworkers produced a variant of protein A by the mutation of amino acids (Falugi *et al.,* 2013). The variant was generated by the substitution of amino acids at residue 9 and 10 or 36 and 37 in all five SpA immunoglobulin-binding domains (IgBDs). These variants were unable to bind to the IgG Fc receptor or to cross-link B cell receptors. When mice were immunized with this mutant SpA, they produced of protein A neutralizing antibodies in mice, conferring protection against *S. aureus* abscess formation. SpA vaccines also enabled the mice to have increased antibody responses against several *Staphylococcus* antigens.

A subunit vaccine has also been developed using *S. aureus* extracellular fibrinogen binding protein (Efb). Efb plays a critical role in *S. aureus* pathogenicity by blocking the phagocytosis of bacteria through a mechanism that links complement protein and coagulase proteins and binding to C3b (Lee *et al.,* 2004, Ko *et al*., 2013). C3b is an opsonin which binds to complement receptors on neutrophils (CR1) activating phagocytosis of the bacterium. *S. aureus* can protect itself from phagocytosis by the secretion of the Efb which attracts fibrinogen at the bacterial surface. This results in the formation of a capsule-like shield. Ko and coworkers (Ko *et al.,* 2013) investigated if the capsule-like shield formed prevents binding of phagocytic receptors to their ligands (C3b and antibody) on the bacterial surface. They first used flow cytometry to analyze whether C3b-labelled bacteria would be detected by fibrinogen binding. They incubated the pre-opsonized *S. aureus* with soluble CR1 in the presence of the fibrinogen and Efb.

They found that in the presence of fibrinogen, Efb prevented the binding of CR1 to bacteria pre-opsonized with C3b. When pre-opsonized bacteria were incubated with Efb and Fg, it disrupted antibody Fc domains, hence the Fc receptors on phagocytes can no longer recognize the bound antibodies. This suggests that the opsonic antibodies directed to *S. aureus* cannot function since Efb hides these antibodies under the Fg shield (Ko *et al.,* 2013).

Clumping factor A (ClfA) is a *S. aureus* adhesion molecule. ClfA has been used in a subunit vaccine (binds to fibrinogen, blood clots, damaged epithelium and platelets). An initial trial with this vaccine was successful, however a phase III trial testing for the ability of the vaccine to eliminating *S. aureus* bacteremia in neonates failed to achieve effectiveness (Reviewed in Daum *et al.,* 2011).

Efforts to find a better method to prevent the spread of this pathogen continue to be tested. To date, only two vaccines have reached the human testing stage, but no data have yet shown that they are effective in controlling the robust spread of *Staphylococcus* infections. StaphVAX™ is a conjugate vaccine that contains *S. aureus* capsular polysaccharide type 5 and type 8. The carbohydrates are coupled to a protein that is known to promote Th2 responses (Fattom *et al.,* 2004). Th2 is a cell produced when a naïve T cells are exposed to IL-4. Alternatively, in the presence of IL-4, IFN-ϒ and IL-2 a naïve T cell can also differentiate to produce Th2. T cells is very important in the generation of opsonizing antibodies, and the Th2 is required for the antibody affinity maturation as well as class switch (Reviewed in Owen *et al.,* 2013). This vaccine candidate was evaluated on 1800 hemodialysis patients to prevent Staph infections. The

patients either received the StaphVAX™ or PBS. This vaccine candidate proved to be safe and produced high antibody levels to CP type 5 and type 8. At an early time point (32 weeks) staph infections were decreased by 64%. Unfortunately, the efficacy of this vaccine lasted for just a year. After one year there were decreased levels of antibodies against both CP type 5 and type 8. (Fattom *et al.,* 2004). Therefore, StaphVAX™ proved to be immunogenic, safe and effective up to the 40th week. However, it only conferred a limited short-term protection against *S. aureus* bacterium. For patients who will require only a one-time immunization, like surgery patients, StaphVAX™ is effective in producing protective antibody levels. However, for patients with a long-term risk of infection, this vaccine could require boosters to increase the levels of the vaccine-specific antibodies, and hence prolong its efficacy (Fattom *et al.,* 2004). A later experiment was conducted to confirm the first Phase III results. It involved 3600 patients who were treated with StaphVAX™ and PBS as the control, and from this the findings indicated that there was no difference between the treated and the controls. Due to these contradictory results the manufacturing of this vaccine was halted (Giersing *et al.,* 2016).

Another *S. aureus* vaccine that has reached the human testing level is the vaccine containing V710. V710 is a highly conserved *S. aureus* protein, called iron determinant B (IsdB). IsdB is required by *S. aureus* in the utilization of hemoglobin as an iron source (Torres *et al.*, 2006). Evidence shows that this protein is immunogenic in mice and rhesus monkeys. Fowler and coworkers conducted a randomized trial to evaluate the efficacy and the safety of V710 in 8031 patients of 18 years and older who

were undergoing cardiothoracic surgery (Reviewed in Fowler *et al.*, 2013). Fowler and colleagues found that there was no significant difference between the treated patients and the placebo groups, and that the group treated with V710 experienced more side effects. This was thought to be because the V710 resulted in increased IgG levels against the IsdB. Multi-organ failures were reported in the course of the study. In addition, more deaths were observed in the treated group. The cause linking V710 antibiodies with multi-organ failure and increased mortality is not yet known. This study was terminated by the independent data monitoring committee.

1.1.5.3.2 Monoclonal antibodies for the treatment of Staphylococcus aureus.

As vaccines have not proven to be effective in the prevention of *S. aureus* infections, immunologists have attempted to produce antibodies that may be used to induce passive immunity. Passive immunity is a form of adaptive immunity that is achieved by the transfer of antibodies from an immune individual to a non-immune individual (Reviewed in Owen et.,al 2013). Antibodies are immunoglobulin proteins secreted by the B cells. They play a role in recognizing a specific epitope on the antigen. As a result, they opsonize pathogens, enhancing phagocytosis and clearance of the antigen (Owen *et al.,* 2013).

Antibodies are traditionally produced by immunizing an animal, through hybridoma technology, or through the use of recombinant DNA methods (Pooja and Ajit, 2010). Hybridomas are formed by the fusion of normal lymphocytes with myeloma cells. This allows the cell to retain the characteristics of antibody production, but also

gives the cell the immortal growth characteristics of myeloma cells. Hybridomas are cloned to make monoclonal antibodies. Monoclonal antibodies are a homogenous preparation of antibody molecules that are produced by a single clone of B cells (Reviewed in Owen *et al.,* 2013).

Antibodies are well known to play a significant role in diagnosis and treatment of disease (Pooja and Ajit, 2010). Antibodies bind to the surface of the bacteria resulting in the elimination of bacteria through neutralization, complement activation, or promoting phagocytosis (Nian *et al.,* 2016). Several disadvantages of treatment with antibodies include the technically difficult procedures involved in preparing antibodies, and a lack of stable, long term responses (Rao *et al.,* 2013). Traditionally prepared monoclonal antibodies are murine (mouse products) used to treat human diseases. This results in an important problem associated with using monoclonal antibodies to treat diseases. The patient forms an immune response to the mouse proteins, producing antibodies that result in removal of the monoclonal antibodies, limiting their efficacy (Nian *et al.,* 2016).

1.1.6 The Phage Display Technology.

S. aureus remains a life-threatening pathogen. Treatment of these infections is complicated due to the resistance of *S. aureus* to multiple antimicrobial compounds and the failure of active and passive immunity to provide protection. There is an urgent need to develop an accurate and rapid method that could identify and control this pathogen. Phage display is a promising technology that could provide tools for the treatment of this disease.

In 1985, phage display technology was introduced by George Smith. A phage is a virus that infects bacteria. The phage can be used as an expression vector, expressing peptides or antibodies on the surface of the phage. Libraries can be constructed with large numbers of phage displayed peptides and protein. These libraries can be used to test for the ability of peptides or antibodies to bind specifically to a ligand. This technology has been applied in the fields of immunology, cell biology, drug discovery and pharmacology (reviewed in Arap 2005).

Willats defined phage display as a technology that allows the presentation of foreign proteins at the surface of the phage particles. The expression of the peptides on the surface of the phage is achieved by incorporating the nucleotide sequence encoding for a peptide or protein into a phage fused to a gene that is encoding a phage coat protein. This fusion ensures that when the phage particle is assembled, the protein of interest is expressed and presented at the surface the mature phage. Numerous phage libraries can be constructed that are then screened for phage displayed proteins that bind to the desired ligand. The process of screening is termed bio-panning or affinity selection. The process involves several steps including, binding, washing, elution and finally amplification. These steps are repeated several times and the process is called the phage display cycle (Reviewed in Willats 2002).

The original phage library having highly diverse protein variants is screened using the phage display cycle, selecting for specific clones. This process achieves this by immobilizing target molecules to a support and then exposing the immobilized target to solutions containing phage. The non-bound phage is removed by washing (Reviewed in

Willats 2002). The washing stage plays a vital role, since it helps to ensure that a balance between specificity and avidity is maintained. Avidity is the strength of binding of more than one receptor to its ligand (Reviewed in Owen *et al.,* 2013). The clones obtained from the phage display libraries may be binding strongly, but with low specificity due to multiple copies of the peptide being displayed on the surface of the phage. If they thoroughly wash the highly specific with weak binding ability may be lost in the process, and if they are not thoroughly washed then the clones obtained may have low specificity with strong binding ability. Therefore, a required balance is achieved by adjusting the washing times and detergent concentrations. The bound phage are then eluted by either increasing the salt concentration or reducing the pH, while avoiding the use of agents that could disrupt disulphide-based links between the supports and targets. Finally, the eluted phage is then amplified by growth in *E. coli*. Thus, phage that is highly specific for the ligand is achieved (Reviewed in Willats 2002).

There are several phage that may be used for phage display, including the Ff filamentous phage, lambda and T7. The most commonly used is the Ff filamentous phage which include the strains M13, f1, Fd. The advantage of filamentous phage is that its size is not restricted, allowing variety in the amount of the DNA that may be added to the phagemid. The Ff bacteriophage is a single stranded DNA (ssDNA) virus that infects several Gram-negative bacteria (Reviewed in Arap, 2005). The Ff filamentous phage employs a non-lytic propagation method, with all of the components of the phage being exported via the bacterial inner membrane. Therefore, the proteins displayed on the phage are limited to those that withstand the exportation. This can be overcome by

using the lytic phage lambda or T7. The Ff bacteriophage still remains the one largely used, since it provides a highly flexible platform (size not restricted) for the display (Reviewed in Willats 2002).

Ff phage are made up of a long cylindrical protein capsid (930 nm in length and 6.5 nm in diameter) containing a single- stranded DNA genome with 6400 nucleotides containing 11 genes. The long cylinder is composed of about 2700 molecules of the 50 amino acid-acid coat protein VIII (pVIII). This phage is two sided, whereby one side has 5 molecules of each 33-residue pVII and 32- residue pIX. The other side has 5 molecules of each 406 residue pIII and 112- residue pVI. The most commonly used protein for peptide expression in many display applications is PIII. Ff phage infects *Escherichia coli* by using pIII binding to the F pilus in *E. coli*. Since Ff phage particles employ non-lytic propagation, *E. coli* secretes phage particles into the growth medium. The process of infection begins when the phage pIII becomes attached to the F pilus of *E. coli*. The circular single-stranded DNA enters the *E. coli* and is taken up by the host DNA replication machinery and is converted into a double-stranded plasmid. The process of replication continues, producing single-stranded DNA which acts as template for the expression of phage proteins. The phage is then assembled by packaging the single-stranded DNA into the coat proteins and the phage is exported through the bacterial membrane (Reviewed in Arap, 2005; Barbas, *et al.,* 2001).

M13 phage are commonly used in the production of phage display libraries. M13 phage is approximately 6.6 nm in diameter, 880 nm in length and it contains 2700 major coat proteins. This phage is non-lytic as it is in the family of Ff phage. It also has two

pairs of the major coat proteins with 3-5 copies each at the ends, whereby one end has the pIII and pVI and the other end has pVII and pIX. Replication in M13 generates numerous copies of the phage particles with approximately 10^{13} pfu/ml produced in small scale cultures (Warner *et al.,* 2014).

1.1.7 Project goals and objectives.

S. aureus is a life threatening pathogen that causes both hospital and community acquired infections. Widespread antibiotic resistance and failure of active and passive immunity to confer the required immunity against staph infections has made it difficult to treat this pathogen. This has posed a global health concern, since there continued increases in the length of stay of infected patients in hospital beds, medical expenses and mortality rates. We therefore hypothesize that the previously selected *S aureus* specific phage clone (MMT5.2) will bind specifically to *S. aureus* type 5 capsule. Our goal is to provide a novel therapeutic approach for the treatment of *S. aureus* infections using these phage clones. Thus, purified capsular polysaccharide *S. aureus* type 5 will be used to test the binding ability and specificity of MMT5.2 in a carbohydrate ELISA. It is hoped that capsule specific peptides produced by the phage may be bactericidal or may be used to target toxins to the surface of the bacterium.

CHAPTER 2.

2.1 MATERIALS AND METHODS.

2.1.1 Materials.

Staphylococcus aureus type 8 strain 49525, *Staphylococcus aureus* type 5 strain 49521, *Escherichia coli* strain ER2738 (All purchased from *American Type Culture Collection, Manassas USA*). Tryptone, Yeast extract, Polyoxyethylene-sorbitan monolaurate (Tween 20), 3,3'5,5-Tetramethylbezidine (TMB), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), Isopropyl-β-D-thiogalactopyranoside (IPTG), Bovine serum albumin (BSA), LB agar, Sodium acetate F.W 136.08, 2,3,5-triphenyltetrazolium chloride (tetrazolium red) F.W 334.8- (all purchased from *Sigma Life Sciences -Sigma-Aldrich St.louis USA*. Sodium chloride F.W 58.44, tetracycline, PEG/NaCl, sterile glycerol, sodium hydrogen carbonate ($NaHCO_3$), non-fat dry milk, DNAse, RNAse, protease K, tris M.W 121.14 Magnesium sulphate ($MgSO_4$) F.W 58.44, all from *Fisher Scientific – Belgium*. Terrific broth, Columbia broth from *Becton Dickinson and company*. M13KE phage, Ph.D 7 phage- Phage display peptide library kit, and HRP/ anti-MI3 Monoclonal peroxidase conjugate (New England Biolabs USA) Casein, sodium periodate ($NaIO_4$) from *Thermo Scientific Rockford, USA*. Sulphuric acid from *Pharmco-AAper USA*. 96 well poly-L-lysine coated plate, dialysis tubing (45mm wide, 12,000-14,000 molecular weight cut off), DEAE- sephacel column.

2.1.2. *Methods.*

2.1.2.1 Preparation of S. aureus type 8 antigen.

Staphylococcus aureus, type 8, was grown in Columbia broth + 2% sodium chloride (NaCl) overnight at 37°C (Thakker et al., 1998). The cells were then washed three times, twice with 10 ml sample buffer (PBS + 1% BSA) the last wash with 10 ml of PBS. Each time the sample was centrifuged in at 13,000 X *g* at 4°C for 30 minutes. After each centrifugation, the supernatant was poured off, and the pellet was vortexed to form a smooth suspension then re-suspended in the wash buffer. The cells were then treated with 3% formalin at room temperature on a rotator overnight. The cells were washed three times as described previously. The cells were then treated with trypsin (1 mg/ml) and incubated at 37°C with shaking overnight at 250 rpm. The cells were again washed three times as described previously and the cells diluted to an optical density of 1.0 at 550 nm (Thakker *et al.,* 1998).

2.1.2.2 Overnight culture of ER2738.

LB broth with 20 ug/ml of tetracyline was inoculated with *E. coli* ER2738 and incubated at 37°C with shaking at 250 rpm. *E. coli* ER2738 was also streaked on an LB agar plate and incubated at 37°C incubator overnight, then stored in the fridge for future use.

2.1.2.3 Phage titering.

A water bath was prepared by pre-warming it to 50°C. LB plates were labelled and then pre-warmed at 37°C for 1 hour. Top agar (LB + 7% Agar) was melted in the

microwave then 3ml of the top agar was dispensed into sterile culture tubes for each expected dilution. To avoid cooling the top agar was maintained at 50°C. The prepared overnight culture of ER2738 (200 µl) was then dispensed into the sterile microfuge tubes, one per the desired phage dilution. To the top agar in the water bath, 2% Xgal and 2% IPTG were added into each sterile microfuge tube (www.bios.niu.edu). Serial dilutions (100- fold) were performed (2 µl of phage into 198 µl of PBS) using sterile conditions. The phage dilution (20 µl) was added to the 200 µl of the overnight culture of *E. coli,* immediately vortexed, and allowed to incubate for 1-5 minutes to allow phage to infect the bacteria. After 1-5 minutes, the cells were added to the 3 ml of top agar containing Xgal and IPTG. The mixture was then quickly and gently poured to the already labelled and pre-warmed LB plates, then swirled gently and allowed to solidify. The same was done to the remaining dilutions. A control plate was also done which contained everything except phage. Thereafter the plates were incubated at 37°C overnight. The plaques were then counted (Barbas *et al.,* 2001, Maniatis *et al.,* 1982).

2.1.2.4 Amplification of phage library.

A colony of *E. coli* ER2738 was inoculated into 20 µl LB tet media and incubated overnight at 37°C with shaking at 250 rpm. Terrific broth (40 ml) with tetracycline media was inoculated with 800 µl of the overnight culture of E. coli ER2738 in a 125 ml flask. The cells were then incubated at 37°C with shaking at 250 rpm for one hour to allow the cells to reach the log phase (OD $_{560\text{-}595}$). The shaking speed was then reduced to 100 rpm for 10 minutes to allow the regeneration of the sheared F pili. Phage (4 X 10^{9}) was added to 40 ml media containing bacteria cells and mixed by swirling. The cells were

then returned to the shaker set at 37°C at 250 rpm for approximately 4-5 hours. To remove the cells, centrifugation was done at 4500 *X g* for 10 minutes. The supernatant was transferred into a new tube, and then centrifuged again. The top 32 ml of the supernatant was transferred to a new tube. An aliquot (500 µl) was saved in a 1.5 ml Eppendorf with 500µl of sterile glycerol then stored in the freezer -20°C. To the remaining solution 8 ml of 2.5 M NaCl/20% PEG/8000 (w/v) was added, then mixed briefly and the phage was precipitated overnight at 4°C.

The following day, phage was centrifuged at 12,000 *X g* at 4°C for 15 minutes. The supernatant was decanted. The pellet was then resuspended in 2 ml TBS, divided into 2 eppendorf tubes and then centrifuged briefly to remove any cell debris. The supernatant was transferred to fresh new tubes. NaCl (2.5 M) containing 20% PEG-8000 (200 µl) was added and incubated on ice for 15-60 minutes. Following this, the tubes were centrifuged at 14,000 rpm on the benchtop microfuge for 10 minutes. The supernatant was discarded. The tubes were centrifuged again and the remaining supernatant was gently removed with a pipette to avoiding disturbing the pellet. The pellet was then resuspended in 200 µl TBS. Finally, 200 µl of sterile glycerol was added and at stored at -20°C for future use (Barbas CF *et al.,* 2001, Maniatis T *et al.,* 1982).

2.1.2.5 Phage ELISA test.

S. aureus, the blank, and M13KE negative control wells were coated with the ligand, 100 µl of formalized and trypsinized *S. aureus* with OD=1 in a poly-L-lysine coated plate. The no *S. aureus* wells were coated with 100 µl of blocking buffer (1% casein in TBS or 5% Non-fat dry milk), all performed in triplicate. Only one positive

control well was used, which was treated with 100 µl of 1 x 10^{10} pfu/ml phage in 0.5M sodium bicarbonate PH 9.6. The plate was incubated at 37°C in a humidified chamber overnight. The plate was then centrifuged at 1200 rpm for 15 minutes, the coating solution was aspirated and the wells washed with PBS once. Each well was then filled with the blocking buffer and incubated at 37°C for 30 minutes. The blocking buffer was then aspirated; and the *S. aureus* test wells and the no *S. aureus* wells were coated with 100 µl of 2 x 10^{11} pfu/ml phage (NL P3 or NL SA.7) in PBS. The negative control wells were coated with 100 µl of 2 x 10^{11} pfu/ml M13KE phage (phage without peptide displayed). The blank and the positive control wells were coated with PBS. The plate was incubated for one hour at room temperature.

The wells were then washed 6 times with the wash buffer (PBS). Each well was then filled with 100 µl of diluted (1:5000 in sample buffer) anti-M13 monoclonal antibody-peroxidase conjugate, then incubated at room temperature for 1 hour. The wells were washed 6 times and 100 µl of TMB substrate solution was added. The plate was incubated up to a maximum of 30 minutes, until a blue color developed. The wells were then treated with the stop solution (50 µl 2N H_2SO_4) and the absorbance was read at 450 nm (Thakker M *et al.,* 1998; Sinha *et al.,* 1999).

2.1.2.6 Growth and harvesting of the S. aureus type 5 bacteria.

Columbia agar was inoculated with *S. aureus*, type 5 bacteria and incubated at 37°C overnight. A colony was used to incubate 330 ml Columbia broth + 2% NaCl in a 1200 ml Erlenmeyer flask at 37°C with shaking overnight. The broth was transferred into centrifuge tubes and centrifuged at 13,000 x g at 4°C for 30 minutes. The pellet was

collected and placed in a 50 ml conical tube. This procedure was repeated until the 50 ml conical tube was full. The paste was then stored at -20^oC (Thakker *et al.,* 1998).

2.1.2.7 Purification of type 5 capsular polysaccharide.

The 50 ml of *S. aureus* type 5 paste was weighed (determined by weighing the conical tube with the paste then subtracting it from an empty conical tube). The cells were suspended at 0.5 g (wet weight) per ml in 0.05 M tris – 2mM $MgSO_4$, P.H 7.5 and autoclaved at 121^0C for 20 minutes. A loop of the suspension was streaked on an LB plate and incubated at 37^0C for 2 days to ascertain that all the cells were dead. DNAse and RNAse (100 µg/ml) each were added and the solution was incubated with shaking at 37^0C and 225 rpm for 6 hours. The solution was treated with protease K (4 units/ ml) overnight. The solution was centrifuged at 17,000 *x g* for 20 minutes at 4^0C. The pellet was discarded and the supernatant was transferred to dialysis tubing (45 mm wide-12,000-14,000 molecular weight cut off) and dialyzed extensively against sterilized water overnight. Dialysis was repeated four times.

When the solution inside the dialysis tubing was clear, the solution was centrifuged at 25,000 *x g* for 30 minutes at 4^0C. The pellet was discarded and the supernatant was filter sterilized using a 0.45 µ filter. Sodium periodate ($NaIO_4$) 0.05M was added to the sample and incubated overnight at room temperature in the dark (the bottle was well covered with aluminum foil and was placed in a dark drawer).

The solution was then transferred to dialysis tubing and dialyzed extensively against sterilized distilled water, as described previously. The sample was transferred to

a 50 ml conical tube and frozen at a slant position (to maximize the surface area) overnight at -20^{0}C. The sample was then transferred to -80^{0}C overnight. The caps were then removed and the tubes covered with Kim-wipes fastened with a rubber band, and then frozen again at -80^{0}C until lyophilization. The samples were removed from the -80^{0}C freezer, immediately placed in the bucket containing dry ice and transported to the lyophylizer. The samples were lyophilized for approximately 2 days. After the samples were dry they were then suspended in 5ml of MilliQ water and stored in -20^{0}C freezer (Fattom *et al.,* 1990, Tzianabos *et al.,* 2001)

2.1.2.8 Regeneration of the DEAE- Sepharose column.

The column bed was washed with 1.5 column volumes of 2 M sodium chloride (NaCl), followed by overnight treatment with 1.5 column volume of 1 M sodium hydroxide to remove bound molecules. The column was then washed with 30% isopropanol by allowing the isopropanol to flow through the column. The column was then washed with 1.5 column volumes of 1 M sodium acetate, pH 3.0 to recharge the column. The column was then re-equilibrated with the starting buffer (0.05 M NaAc + 0.1 M NaCl pH 6.0) (Fattom *et al.,* 1990).

2.1.2.9 Chromatography and fraction collection.

The column was washed with 2 X the bed volume with starting buffer. The column was then allowed to run until approximately 3mm of fluid was left on the top of the DEAE sepharose solution. Buffer with the carbohydrate sample (6ml) was slowly added to the top of the column. The column was washed with 5 column volumes of

starting buffer. The solution was changed to elution buffer (0.05 M NaAc + 0.15 M NaCl pH 6.0) and the column was allowed to run simultaneously as the fraction collector was turned on, set to collect approximately 7ml per fraction of the carbohydrate solution (Cooper 1977).

2.1.2.10 Carbohydrate test: Red tetrazolium (2,3,5- triphenyltetrazolium chloride).

Carbohydrate solution (200 µL) was placed in a clean test tube, then 200 µl of 10% Sodium hydroxide (NaOH) solution was added and finally 1 ml of 0.5% aqueous solution of red tetrazolium was added. This was done in triplicate. The tubes were placed in a beaker with hot boiling water on a heating plate, and then the development of color was observed. A negative control was distilled water and positive control was 1 mg/ml of glucose. Absorbance of the samples was read at 490 nm. A standard curve performed using a two-fold serial dilution of 1mg/ml glucose which was used to produce a linear regression line. The linear regression line was used to determine the amount of carbohydrate in each positive pool (Fieser et al, 1987).

2.1.2.11 Protein concentration test: Bradford assay.

A standard curve was created using 20 µl of 1 mg/ml BSA in the first well of a 96 well plate. A two-fold serial dilutions (20 µl ddH_2O and 20 µl BSA) was performed to the next wells. Bradford reagent (200 µl) was placed in each well needed for the standard curve, blank and the peaks. The samples were done in duplicate. Carbohydrate positive peaks (20 µl) were added to the well, the blank and negative control received 20 µl of MilliQ water and elution buffer respectively (Bradford 1976).

2.1.2.12 Phosphate test for Teichoic acids.

Pooled carbohydrate fraction (200 µl) each were dissolved in 0.5 ml 2 M HCL to release phosphate from teichoic acid. The samples were placed in boiling water bath for 30 minutes. The volume was then adjusted to 4 ml with distilled water. Reagent C (4 ml of 1 volume of 6 N sulfuric acid with 2 volumes of distilled water, 1 volume of 2.5% ammonium molybdate, and 1 volume of 10% ascorbic acid) was added into each tube. Tubes were capped with Parafilm, mixed gently and placed in a rack at 37^0C incubator for 2 hours. After 2 hours, the tubes were taken out of the incubator and observed for color change. Absorbance was read at 820 nm using ultraviolet spectrophotometer (Chen *et al.,* 1956).

2.1.2.12 DNA concentration test.

Using an ultraviolet spectrophotometer, the absorbance of the elution buffer (blank) and the sample was read at 230 nm, 260 nm, 280 nm, and 320 nm. The concentration of DNA in the samples was then determined (Sambrook and Russell 2001).

2.1.2.13 ELISA for S. aureus capsular polysaccharide.

A poly-L lysine plate was coated with 100 µl antigen (S. aureus CP), the positive control well was coated with the whole bacteria SAT5, and the blank coated with PBS. The plate was left overnight at 37^0C incubator. Solution was removed from the plates by flicking plates over the sink and blotting on the paper towel. Unreacted protein-binding sites on the wells were blocked by incubating samples with 150 µl 1% casein for 30

minutes at 22^{0}C. Wells were washed twice with PBS containing 0.05% Tween-20. After the wash, the blank well received 100 µl sample buffer (PBS with 0.1% BSA and 0.05% Tween-20). A 1:1000 dilution of (100 µl) antiserum was added to the positive control well. Monoclonal antibody BN.T5.A2 supernatant was added to the rest of the wells. The plate was incubated at 37^{0}C for 2 hours to allow binding. Wells were washed as previously described. To all wells, 100 µl of 1:1000 dilution in sample buffer of anti-mouse Ig-PO was added. Wells were allowed to bind for 30 minutes at 37^{0}C. Wells were washed as previously described. TMB solution was added to all wells, incubated for 30 mins at 22^{0}C till there was a color change. H_2SO_4 (50 µl of 2 N) was added to each well to act as a stop solution. Absorbance of each well was read at 450 nm (Thakker *et al.,* 1998, Sinha *et al.,* 1999).

2.1.2.14 Carbohydrate ELISA.

To a 96-well tissue culture plate, the capsule wells (CP), and blank were coated with 100 µl purified capsular polysaccharide type 5, No capsule wells (NO CP) were coated with 100 µl PBS. All were performed in duplicate. Only one positive control well was used, which was treated with 100 µl of 1.75 x 10^{11} pfu/ml M13KE phage in 0.5 M sodium bicarbonate pH 9.6. The plate was covered with a plastic wrap and incubated at 37°C for 30 minutes in a humidified chamber. The coating solution was aspirated and the wells washed with 200 µl PBS once. Each well was then filled with the 200 µl blocking buffer (5% nonfat dry milk) and incubated at 37°C for 30 minutes. Blocking buffer was then aspirated, and CP and No CP wells coated with M13KE, NLT8.7 and

MMT5.2. The blank and the positive control wells were coated with sample buffer (PBS + 0.1% BSA). The plate was incubated for one hour at room temperature.

The wells were then washed 6 times with the wash buffer (PBS). Each well was filled with 100 µl of anti-M13 –PO diluted 1:5000 in sample buffer (PBS + 5% NMS) and then incubated at room temperature for 1 hour. The wells were washed 6 times and 100 µl of TMB substrate solution was added. The plate was incubated up to a maximum of 30 minutes, until a blue color developed. Wells were then treated with the stop solution (50 µl 2 N H_2SO_4) and the absorbance was read at 450 nm (Thakker M *et al.,* 1998; Sinha *et al.,* 1999).

CHAPTER 3.

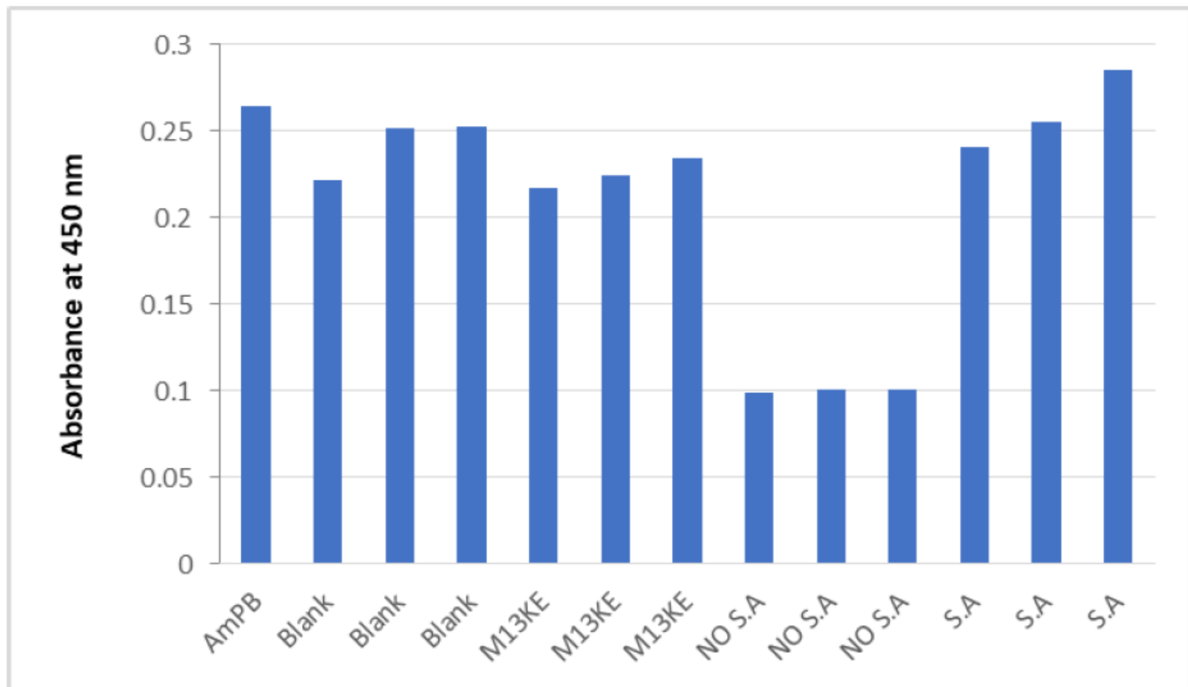
3.1 RESULTS.

3.1.1 Preparation of S. aureus whole cell antigen.

Staphylococcus aureus, type 8, was grown overnight at 37°C in Columbia broth + 2% sodium chloride (NaCl) (to allow only *S. aureus* to grow and inhibit other bacteria, since *S. aureus* is salt tolerant). The cells were treated with 3% formalin (to kill the bacteria). Cells were then washed and treated with trypsin (to remove the Protein A, which causes non-specific binding to the antibodies). Finally, cells were washed and diluted to an optical density of 1.0 at 550 nm. The cells were then used to determine if phage (NLT8.7) bound specifically to *S. aureus*, type 8 using an ELISA assay.

3.1.2 Test of ELISA negative controls.

There were many problems with the initial assay (Figure 1). The positive control was too low. Positive controls usually have an absorbance of over 2.0. In addition, the negative controls (blanks and M13KE) were too high. The blank containing no phage was as high as the positive control. This high absorbance with no phage and no ligand indicates nonspecific binding of antibody to the plate. Our SA wells were also high (with no added phage), indicating that *S. aureus* type 8 had a high background due to non-specific binding to antibody.



Blank: no phage, S.A, Ab. NO SA: phage, Ab.
M13KE: M13, Ab, S.A. S.A: phage, S.A, Ab

Figure 1: NLT8.7 tested for binding to *S. aureus* type 8. A 96 well poly-L-lysine coated plate was incubated with formalized and trypsinized *S. aureus* type 8 (SA). The Positive well received AmPB (2.8 X 10^{12} pfu/ml) phage library diluted in 0.05 M $NaHCO_3$, pH 6. The plate was incubated overnight at 37^0C and centrifuged at 400 x g. Wells were washed with PBS and blocked with PBS + 1% BSA blocking buffer. Peptide phage (NLT8.7) and M13KE (phage without peptide) were diluted in sample buffer containing 0.1% BSA in PBS. Peptide phage (NLT8.7) was introduced into test well (SA) and No S.A wells. Blank received sample buffer instead of phage. After incubation and washing, an anti-M13 antibody-conjugated to horseradish peroxidase (HRP) was added to all wells. Wells were again incubated and washed, then TMB substrate was added. Color development was determined by measuring absorbance at 450 nm.

3.1.3 Test of blocking buffer role in non-specific binding.

We determined that our low positive control was due to low phage titer in M13KE. To increase the amount of phage added, the sodium carbonate buffer was concentrated tenfold, allowing the addition of more dilute phage to the well. This resulted in normal absorbance in our positive control (Figure 2). We also addressed the problem of high nonspecific binding. Blocking buffer is used to prevent non-specific binding of the antibody to the plastic. To determine whether our block was allowing non-specific binding of the antibody to the plastic, we changed the blocking buffer from PBS + 1% BSA to nonfat dry milk and the sample buffer from PBS + 0.1% BSA to PBS + 5% NMS for antibody and PBS + 0.1% BSA for phage. We then tested the binding specificity of the previously selected clones (NLT8.7 and MMT5.2) to the presence or absence of *S. aureus* type 5 or *S. aureus* type 8) using an ELISA assay (Figure 2). When comparing NLT8.7 and MMT5.2 binding to wells containing SA to wells containing no ligand, the results were significantly different ($p < 0.01$). However, the blank and negative controls (M13KE) were as high as the wells containing phage and SA. This high absorbance indicates continued nonspecific binding of antibody to the plate.

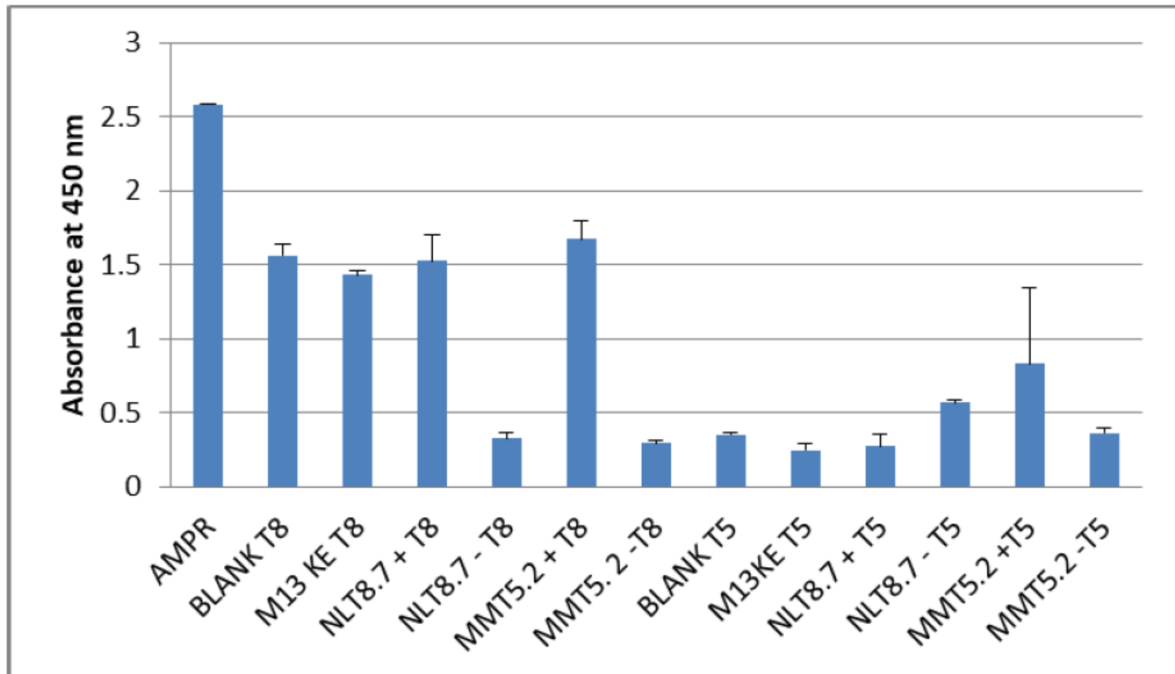


Figure 2: Phage Elisa with the previously selected clones NLT8.7 and MMT5.2 for binding in presence and absence of *S. aureus* type 5 (T5) and type 8 (T8). A 96 well poly-L-lysine plate was coated and incubated with formalized and trypsinized S. aureus or PBS. Positive wells received AmpR diluted in 0.05 M $NaHCO_3$ pH 9.6. The plate was incubated overnight at 37^0C and centrifuged at 400 x g. Wells were washed with PBS and blocked with nonfat dry milk (NFDM). Phage clones (NLT8.7 and MMT5.2) and M13KE were diluted in sample buffer (PBS + 0.1 BSA). Phage clones were introduced into all wells, except M13KE (M13 with no peptide) and Blank (received sample buffer instead of phage). After incubation and washing steps, an anti-M13 antibody which was conjugated to horseradish peroxidase (HRP) in sample buffer (PBS + 5% NMS) was added to all wells. Wells were again incubated and washed. TMB substrate was added. Color development was determined by measuring the absorbance at 450nm. Data is represented as the mean +/- SD for triplicate observations. Significance was determined by a T Test (** = $p < 0.01$).

3.1.4 Test of the role of protein A in non-specific binding.

To determine whether our *S. aureus* type 8 was nonspecifically binding due to presence of Protein A, we retrypsinized (treating *S. aureus* again with trypsin to ensure that we removed all of the protein A) the bacteria and repeated the ELISA assay. Retrypsinization greatly improved the background in *S. aureus* type 8 (*Figure 3*). However, absorbance in the negative control and blank was still as high as the absorbance seen with phage binding to *S. aureus*. Due to the difficulties observed with the whole cell bacteria, the ELISAs were repeated with purified capsular polysaccharide. To perform these assays required the preparation of purified capsule.

3.1.5 Purification of capsular polysaccharide type 5.

S. aureus type 5 paste was collected and suspended in 0.05M tris-2mM MgSO4 autoclaved. The paste was treated with DNAse (to remove DNA), RNAse (to remove RNA) and incubated. Protease K (to remove Proteins) was then added. The solution was then incubated overnight in the dark at room temperature with sodium periodate (to remove teichoic acids). The solution was extensively dialyzed against sterile MilliQ water and then lyophilized. The sample was dissolved in sodium acetate and NaCl and DEAE sepharose column chromatography was performed. The column was washed, and the carbohydrate was eluted with elution buffer (0.05M sodium acetate + 0.15M NaCl, pH 6.0) and 7 ml fractions were collected into 80 tubes. A red tetrazolium carbohydrate assay was performed on all fractions collected to determine the presence of the carbohydrate. Six peaks were obtained which include P1 (1-23), P2 (24-45), P3 (46-54), P4 (55-62), P5 (63-69) and P6 (70-80) (Figure 4).

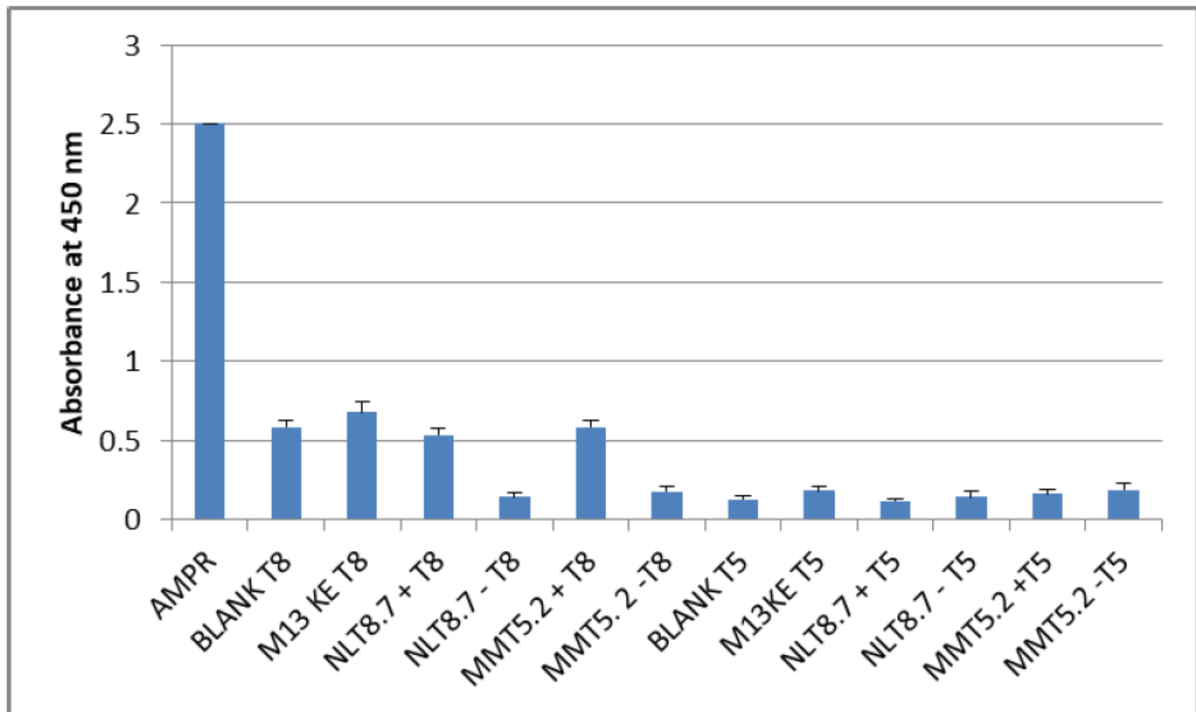


Figure 3: Phage Elisa with the previously selected clones NLT8.7 and MMT5.2 for binding to retrypsinized *S. aureus* type 5 (T5) and type 8 (T8). A 96 well poly-L-lysine plate was coated and incubated with formalized and retrypsinized *S. aureus* or PBS. Positive wells received AmpR diluted in 0.05m $NaHCO_3$ pH 9.6. The plate was incubated overnight at 37^0C and centrifuged at 400 x g. Wells were washed with PBS and blocked with nonfat dry milk (NFDM). Phage clones (NLT8.7 and MMT5.2) and M13KE were diluted in sample buffer (PBS +0.1% BSA). Phage clones were introduced into all wells, except M13KE (M13 with no peptide) and Blank (received sample buffer instead of phage). After incubation and washing steps, an anti-M13 antibody which was conjugated to horseradish peroxidase (HRP) was added to all wells. Wells were again incubated and washed. TMB substrate was added. Color development was determined by measuring the absorbance at 450 nm. Data is presented as the mean +/- SD for triplicate observations. Significance was determined by a T Test (** = $p < 0.01$).

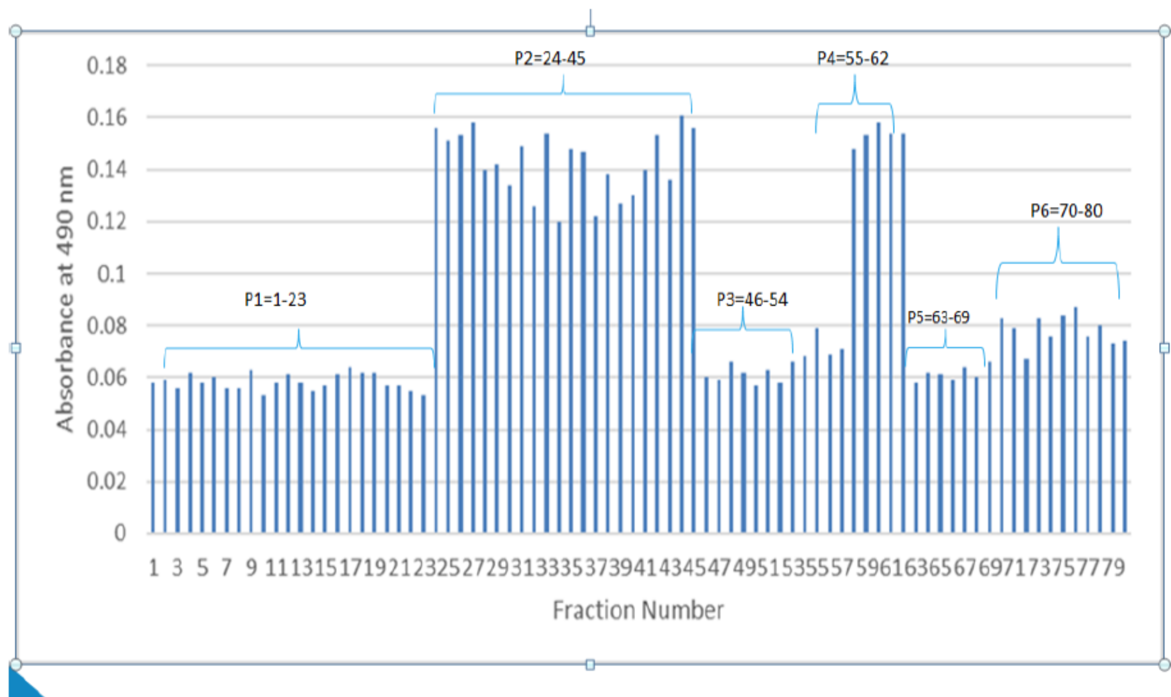


Figure 4: Carbohydrate assay of DEAE column fractions using a Red tetrazolium reagent. *S. aureus* paste (50 ml) was autoclaved with Tris-$MgSO_4$, then treated and incubated with DNAse, RNAse, protease and $NAIO_4$ to remove non- carbohydrate contaminants. The solution was dialyzed and lyophilized then dissolved in 0.05 M Sodium Acetate + 0.1 M NaCl and added to a DEAE sepharose column. The column was then eluted with 0.05M sodium acetate and 0.15 M sodium chloride. Fractions (7ml) of the solution were collected into 13 X100 mm tubes. From each tube 200 µl of the solution was put into a new glass tube, and mixed with Red tetrazolium (1 ml) and 200 µl of NaOH. The tubes were boiled until a color change developed and 100 µl of each sample was added to a microtiter plate and the absorbance was read at 490 nm. Six peaks were obtained which include P1 (1-23), P2 (24-45), P3 (46-54), P4 (55-62), P5 (63-69) and P6 (70-80).

These peaks were further tested for determination of carbohydrate concentration using the red tetrazolium assay. The positive control was a known glucose concentration solution (1mg/ml) which was used to perform a 2-fold serial dilutions. This solution was used to obtain a red tetrazolium standard curve, the linear regression gave an equation $y= -0.0558x + 0.5764$. The R^2 obtained showed a strong correlation ($R^2= 0.908$). The equation was used to obtain the concentration of carbohydrate in the peaks (Figure 5, Table 2).

3.1.6 Protein concentration test using the Bradford Reagent.

Bradford reagent was utilized to determine the protein concentration of any contaminants in our purified carbohydrate (Figure 6). Bovine Serum Albumin (BSA) was used as a positive control. Concentrations of 1-5 mg/ml were used to produce a standard curve. The negative control was the DEAE elution buffer. A linear regression equation ($Y= -0.0821X + 0.5411$) obtained from the dilutions was used to determine protein concentrations in all the peaks obtained (Table 2). From the results, the protein concentrations were outside the range of the standard curve, hence it was not detectable.

3.1.7 Phosphate test for teichoic acid.

A phosphate assay was used to determine the presence or absence of teichoic acid, which is a contaminating carbohydrate that may be found in the DEAE eluate. Acid hydrolysis is used to liberate phosphate from teichoic acid. Phosphate buffered saline (PBS) 1X was used a positive control, elution buffer as negative control. Comparing the values from the pools to the negative controls, samples 2-6 were as low as the negative control. These results indicate that the presence of teichoic acid was negligible (Figure 7). However, pool 1 showed evidence of possible teichoic acid contamination, although slight.

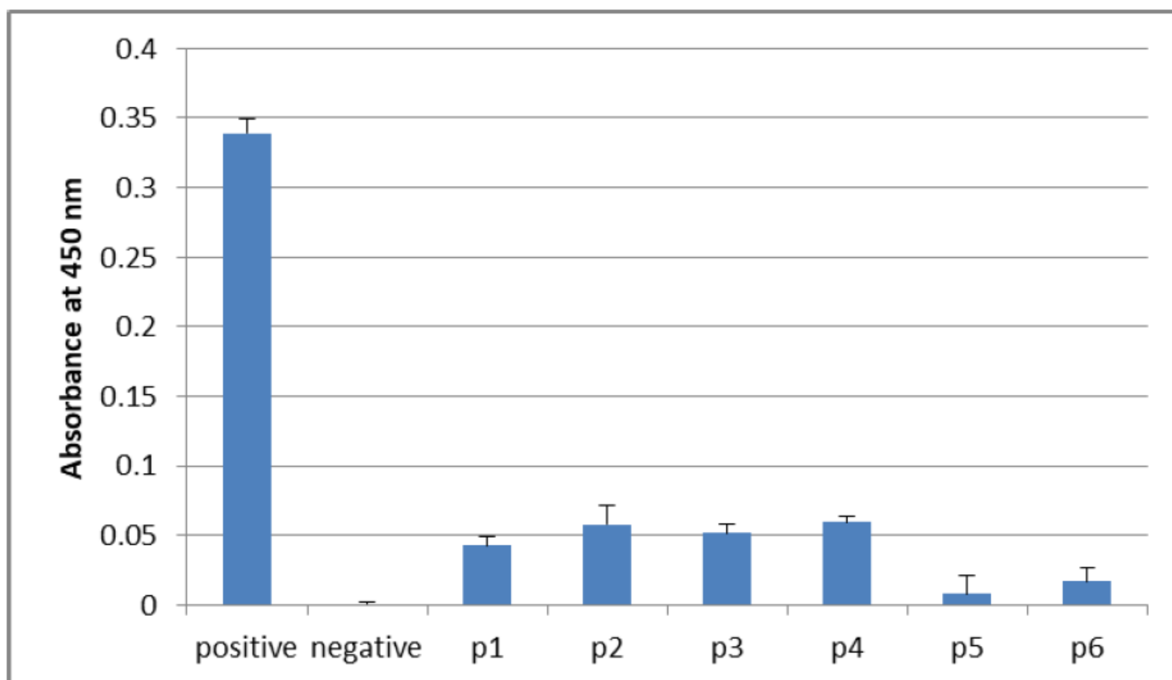


Figure 5: Red tetrazolium carbohydrate test of the pooled peaks. A red tetrazolium reagent was utilized to determine the concentration of the carbohydrate in the peaks obtained. The positive control was a known concentration (1mg/ml) of glucose. The negative control was MilliQ water. To each, sample 1 ml of Red tetrazolium and 200 µL of NaOH was added. The tubes were boiled until a suitable red color developed. Then 100 µl of each sample was put into a microtiter plate. The absorbance was read at 490 nm. The data is expressed as the mean +/- SEM of triplicate observations.

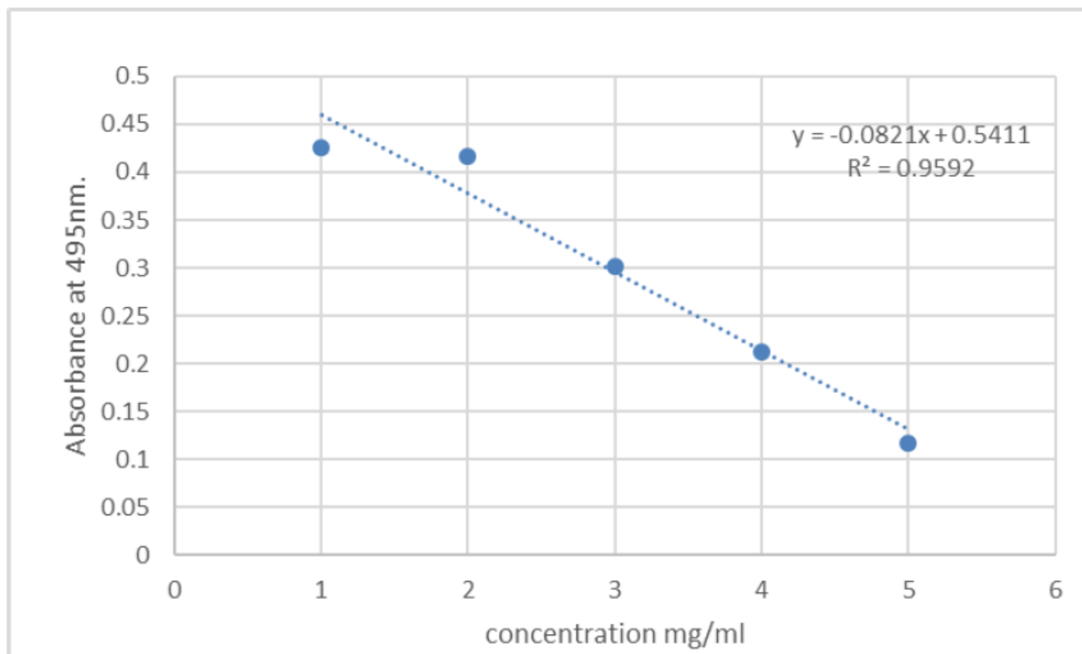


Figure 6: Protein assay of carbohydrate peaks using the Bradford reagent. To a 96-well plate, 100 µl BSA at concentrations of 5 mg/ml, 4 mg/ml, 3 mg/ml, 2 mg/ml and 1 mg/ml were added. To the positive samples (BSA), negative control (MilliQ water) and the pooled peaks, Bradford reagent was added. The plate was mixed for 5 seconds. Absorbance was read at 595 nm. Data was performed in triplicate. The linear regression obtained from the standard curve was used to determine the protein concentration in the pooled carbohydrate peaks as shown in the summary of purification test (Table 1).

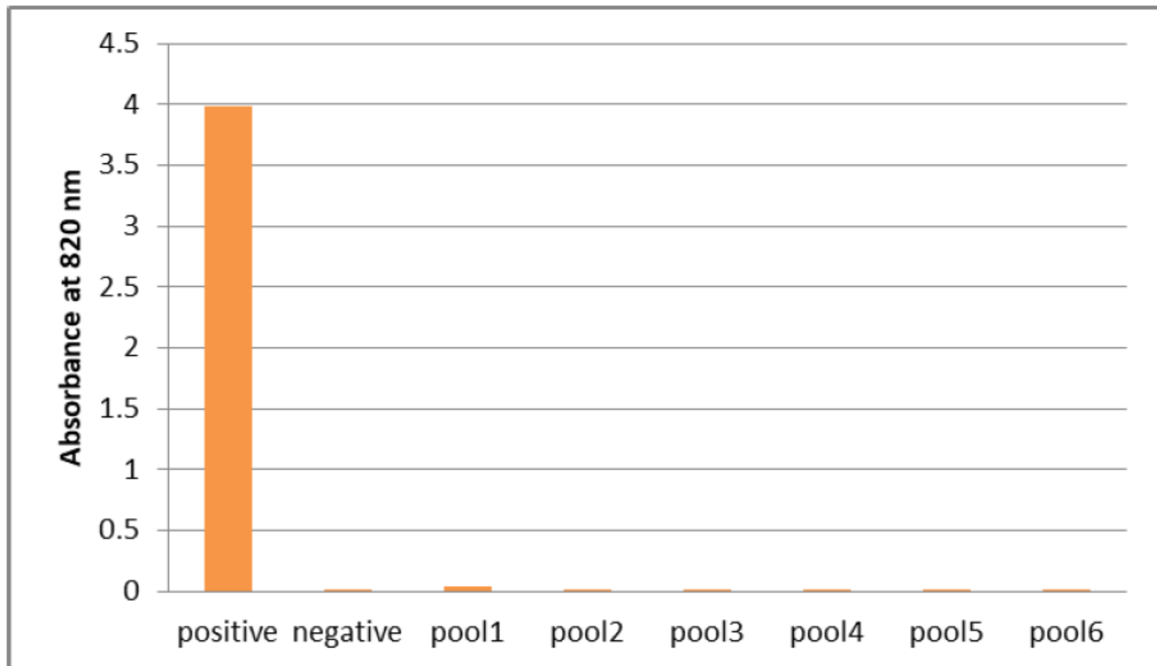


Figure 7: Phosphate test for teichoic acids: To the positive (PBS), negative (MilliqQ water) and the carbohydrate pools, phosphate was released from teichoic acid by dissolving the samples in 2 M HCl. Tubes were boiled for 30 minutes, after which reagent C (1 volume 2.5% ammonium molybdate, 1 volume 10% ascorbic acid, 1 volume 6 N sulfuric acid and 2 volumes of distilled water) was added. Tubes were incubated at 37^0C for 2 hours. Absorbance was read at 820 nm. Data is expressed as the mean +/- SEM of triplicate observations.

Table 1: DNA concentration test.

Sample[a]	230nm	260nm	280nm	320nm	dsDNA µg/ml	A260/A280	A260/A230
P1	0.068	0.004	0.002	0.002	0.24	2.000	0.070
P2	0.032	0.005	0.005	0.009	0.27	0.915	0.164
P3	0.058	0.026	0.019	0.016	1.30	1.368	0.447
P4	0.044	0.026	0.019	0.018	1.32	1.368	0.595
P5	0.064	0.028	0.020	0.016	1.43	1.373	0.443
P6	0.059	0.031	0.020	0.012	1.56	1.493	0.525

[a]The absorbance of the buffer (elution) and the samples (p1- p6) was measured at 230nm, 260nm, 280nm, and 320nm – using a spectrophotometer. The presence of DNA was determined by A260/280 ration = 1.7-2.0 and A260/ A230 ratio > 1.5.

Table 2: showing summary of all the purification tests.

Sample	Carbohydrate mg/ml	Protein mg/ml	Teichoic acid	A260/A280
Pool 1	10.66	0.0065	0.0479	2.000
Pool 2	9.29	0.0065	0.0012	0.915
Pool 3	9.42	0.0065	0.0016	1.368
Pool 4	9.27	0.0009	0.0008	1.368
Pool 5	10.20	0.0065	0.0023	1.373
Pool 6	10.04	0.0065	0.0003	1.493

3.1.8 DNA concentration test.

The presence or absence of nucleic acids (DNA and RNA) was determined by measuring the absorbance of the samples using a spectrophotometer at 230 nm, 260 nm, 280 nm, and 320 nm. The DNA concentration was calculated using the equation DNA µg/mL = A_{260} X 50. The contaminating DNA should have a A_{260}/ A_{280} ration of 1.7-2.0 and a A_{260}/A_{230} ration greater than 1.5. From our results, only P1 had the presence of DNA (A_{260}/A_{280} = 2). Peak 2- 6 showed absence of DNA A260/A280 ranging from 0.915-1.493. A260/ A230 had lower values less than 1.5 (Table 1).

3.1.9 Carbohydrate ELISA assay for specificity of NLT8.7 and MMT5.2 to S. aureus type 5 capsule.

To determine whether the NLT8.7 and MMT5.2 phage could specifically bind to our pooled purified capsular polysaccharide *S. aureus,* type 5, a carbohydrate ELISA was performed. A 96-well cell culture plate was coated PBS (No CP wells), and purified capsular polysaccharide (CP wells). Blanks were also coated with the ligand (CP). The positive control contained M13KE bound to the plate using sodium bicarbonate buffer. The plate was incubated overnight. Wells were blocked with 5% nonfat dry milk. Wells were washed then the No CP and CP wells were coated with M13KE, NLT8.7 or MMT5.2. After three washes, wells were incubated with anti-M13 Ig-PO in sample buffer. After incubation wells were washed, and TMB substrate was added and the plate was read 450 nm. Comparing the no capsule wells to the capsule wells in NLT8.7 and MMT5.2, no increased binding was seen in the presence of carbohydrate (Figure 8).

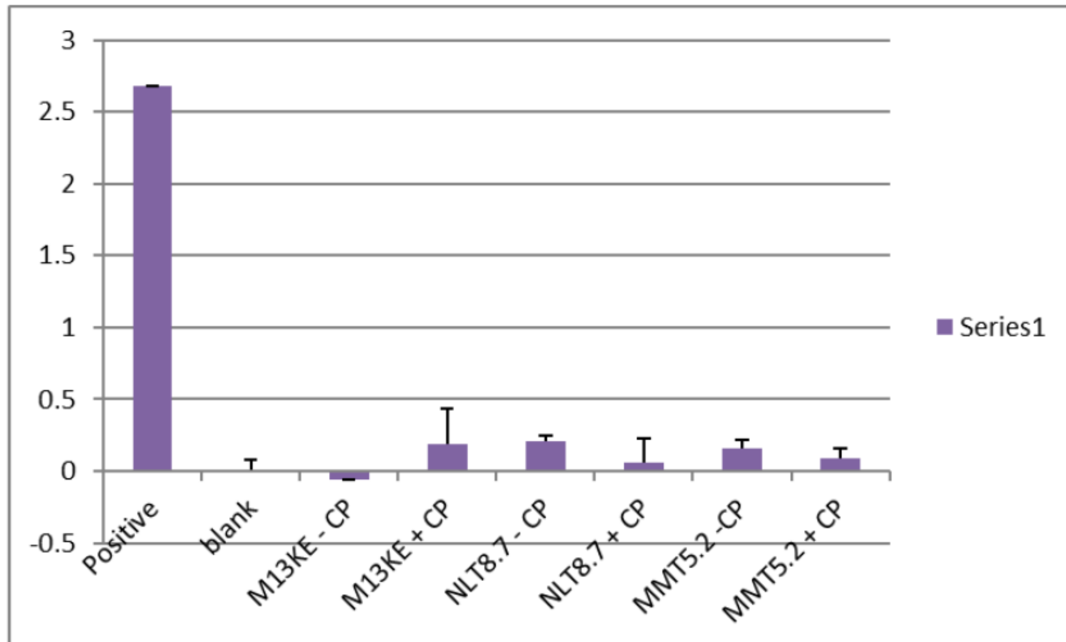


Figure 8: ***Carbohydrate ELISA assay for specificity of NLT8.7 and MMT5.2 to S. aureus type 5 capsule.*** A 96-well cell culture plate was coated with PBS (No CP wells) or purified capsular polysaccharide (CP wells). Blanks were also coated with CP, but received no phage, positive control was M13KE bound to the plate using 0.5 M sodium bicarbonate, pH 9.6. Wells were blocked with 5% nonfat dry milk. Wells were washed and the No CP and CP wells were coated with M13KE, NLT8.7 and MMT5.2. After a wash, wells were incubated with anti-M13 Ig-conjugated to PO in sample buffer. After incubation, wells were washed and TMB substrate was added. Absorbance was read 450 nm. Data is expressed as the mean +/- SD for duplicate observations.

CHAPTER 4.

4.1 DISCUSSION.

In our study, we hypothesized that the previously selected *S.aureus* specific phage clones (MMT5.2) will bind specifically to *S. aureus* type 5 capsular polysaccharide. Previous study (Lenkey 2016), observed high background information while working with *S. aureus* type 8 whole cell bacteria in ELISA assays. We worked on reducing the background information in *S. aureus* type 8 whole cell bacteria. We thought that the non-specific binding was due to inappropriate blocking buffer. We changed the block and repeated ELISA assays. We still obtained high background due to non-specific binding of the antibody to the plate. We thought maybe non-specific binding could be due to protein A, we retrypsinized our bacteria and repeated the ELISA assay, however, no positive result was observed.

Due to the difficulties we observed while working with whole cell bacteria, we decided to repeat our ELISAs with purified capsular polysaccharide. Capsule polysaccharide type 5 and 8 are expressed in most (90-99%) of *S. aureus* isolates in the hospital. Capsule acts as a virulence factor that protects against killing of the bacterium from phagocytosis (Nanra et al., 2013, Nelles et al., 1985, Thakker et al., 1998). We purified the capsular polysaccharide *S. aureus* type 5, determined the presence of the carbohydrate using a red tetrazolium, determined the purity of the sample and then, we performed a carbohydrate ELISA assay for specificity of NLT8.7 and MMT5.2 to *S. aureus* type 5 capsule. MMT5.2 showed no increased binding in the presence and absence of carbohydrate. This shows that our findings did not support our hypothesis.

Our study focuses on the use of phage display technology to select peptides that could specifically bind to *S. aureus,* type 8 and type 5. Phage display technology is a very powerful technology in which a protein is bound to a phage peptide and displayed on the phage surface. The DNA sequence encoding the peptide is contained within the phage. Phage libraries having billions of displayed peptides and proteins (Mullen *et al.,* 2006). Phage display uses bio-panning to select peptides that bind specifically to a target (Reviewed in Williants 2002).

In our study, we hypothesized that the previously selected *S. aureus* specific phage clones (MMT5.2) will bind to *S. aureus,* capsular polysaccharide. In previous studies (Lenkey, 2016; Maratani, 2017), phage display was used to select for phage displaying peptides specific for S. aureus, type 5 and type 8. These studies used the PhD-7 library (New England BioLabs) and panning against *S. aureus*, type 5 and type 8, to select for peptides that bound to *S. aureus*.

In the current study, the previously selected phage clones NLT8.7 and MMT5.2 were tested for specificity to *S. aureus,* type 8 and type 5, using an ELISA assay (Figures 1 and 2). A poly-lysine microtiter plate was coated with the ligand (*S. aureus* type 8). Blanks wells received everything except phage. Blank served to check whether the antibody (HRP/ anti-M13 Monoclonal Peroxidase conjugate is binding to *S. aureus*. M13KE was used as a negative control. M13KE phage has no peptide, the purpose of these wells was to determine whether M13 is binding nonspecifically to *S. aureus*. The No S.A wells received the peptide phage, antibody but no *S. aureus*. The purpose was to

determine if the peptide phage (NLT8.7) was binding to anything else (blocking buffer or the plastic wells) rather than *S. aureus*. SA wells received everything (peptide phage, *S. aureus* type 8 and the antibody), the purpose was to investigate if the phage was specifically binding to *S. aureus*. The plate was incubated, washed to remove the unbound phage, then HRP conjugated anti-M13 antibody was added. Horseradish peroxidase is an enzyme that acts on the tetramethyl benzidine (TMB) substrate to produce a color change. Once a suitable color was formed stop solution (2N H_2SO_4) was added to prevent further reaction by inactivating the enzymes. The absorbance was then read using a spectrophotometer at 450nm. We observed high background due to non-specific binding of antibody to the plate or to *S. aureus* type 8 (Figures 1 and 2).

The non-specific binding in our results could be due to the use of an inappropriate block. Non- specific binding of the ligand to the plate can be prevented by saturating the unoccupied binding sites with blocking buffer. Blocking buffer is efficient if it improves the sensitivity of an assay by reducing the background signal. Bovine serum albumin (BSA) is frequently used by scientists as a blocking agent to prevent nonspecific binding of the antigens and antibodies to the plate. Our previous ELISAs (Figure 1) used BSA as the blocking agent. Normally, a concentration of 1-3% is used (Gibbs 2001). Under certain circumstances, BSA (serum protein) can cause non- specific ELISA signals. Xiao and coworkers (Xiao and Isaacs, 2012), described a non-specific binding interaction which occurred between a vaccinia virus complement control protein (VCP) to human C3b and C4b found in some preparations of BSA. When they used BSA that was prepared in the lab (BSA-2939- globulin free and endotoxin low),

instead of commercial BSA, the nonspecific binding was not observed. Therefore, to prevent false positive results because of cross reactive antibodies or non-specific binding of ELISA reagents to BSA, alternative blocking agents can be used (Xiao and Isaacs 2012).

The blocking buffer in these studies was changed from PBS + 1% BSA to 5% non-fat dry milk (NFDM) (Figure 2). NFDM has the potential to deteriorate if not properly prepared and stored. However, it is an excellent block because of its molecular diversity and amphipathic properties (Gibbs 2001). Duhamel and Johnson (Duhamel and Johnson 1985) used non-fat dry milk to block nonspecific nuclear and membrane staining by avidin conjugates. They proved that a solution of 5% (w/v) non-fat dry milk in buffered saline was the most effective block. They also found that a solution of 2.5% (w/v) was also effective in blocking the background reactivity in the analysis of membrane fluorescence using fluorescence activated cell sorter (FACS).

Even with NFDM block, high non-specific binding was observed in our ELISA using the ligand *S. aureus* type 8 whole cells with the selected clones (NLT8.7 and MMT5.2) (*Figure 2*). Since we also obtained high non-specific binding in our negative controls, we thought that the non-specific binding could be because of protein A. Protein A is found on the surface of 99% of coagulase- positive Staphylococci. Protein A binds to the Fc region of IgG. Thus, binding of protein A on the surface of *S. aureus* to the Fc region of IgG (anti-M13 antibodies) results in high background in an ELISA assay. Several methods have been proposed for reducing interference caused by protein A. Guidry and coworkers investigated the effect of trypsin on specific binding in Becker's *S.*

aureus. Trypsin (1 mg/ml) was added to formalin-killed organisms before adding it to a microtiter wells for ELISA assay. The purpose of trypsin treatment was to remove protein A, which significantly reduced specific binding of monoclonal antibody in the ELISA (Guidry et al., 1991). Since our samples were already trypsinized, we decided to retreat them with trypsin again. This greatly improved non-specific binding in *S. aureus* type 8, but still we had trouble with our negative controls which showed high backgrounds (*Figure 3*).

Due to the difficulties we observed while working with whole cell bacteria, we repeated our ELISAs with purified capsular polysaccharide. We focused on capsule specific phage for two reasons. Capsule polysaccharide type 5 and 8 are expressed in most (90-99%) of *S. aureus* isolates in the hospital setting. In addition, the capsule acts as a virulence factor that protects against killing of the bacterium from phagocytosis (Nanra et al., 2013, Nelles et al., 1985, Thakker et al., 1998). For these studies we purified the capsular polysaccharide S. aureus type 5, determined the presence of the carbohydrate using a red tetrazolium assay.

To determine the purity of the capsular polysaccharide *S. aureus* type 5, protein in the sample was measured with Bradford reagent (Figure 6), the presence of teichoic acids was determined using phosphate test (Figure 7), and the presence of nucleic acids was determined by measuring absorbance at 230nm, 260nm, 280nm, and 320nm (Table 1). Pool 1 contained 20-fold more protein than the other fractions and had an A260:280 ratio of 2.0, suggesting contamination with nucleic acid (Table 2). Pool 1 was excluded

from further analysis and the remaining pools were combined and used as purified capsular carbohydrate.

Phage clones NLT8.7 and MMT5.2 were tested for binding to S aureus, type 8 capsular carbohydrate using an ELISA (Figure 8). Previous tests with these phage clones showed that NLT8.7 bound specifically to whole cell S. aureus, type 8, but did not bind to purified type 8 capsule (Lenkey, 2016). This clone was used as a negative control for binding to capsule. MMT5.2 was demonstrated to bind to whole cell *S. aureus,* type 5, but did not bind to *S. aureus,* type 8, and suggesting specificity for capsule. This clone was not previously tested for binding to purified type 5 capsules. Results for NLT8.7 (Figure 8) did not indicate a significant difference in the presence or absence of carbohydrate. Unfortunately, MMT5.2 also showed no significant binding to carbohydrate. As neither clone showed increased binding in the presence of carbohydrate, our findings with these clones did not support our hypothesis.

Phage display technology has been exploited by other laboratories in the study of infectious diseases. In malaria research, Lauterbauch and coworkers used this technology to determine the host-pathogen interactions between humans and *Plasmodium falciparum*. *P. falciparum* is the causative agent of malaria. A *P. falciparum* cDNA phage display library was panned against immobilized human erythrocyte membrane proteins. Seven parasite proteins are involved in the invasion and exit from human erythrocyte by *P. falciparum*. These proteins could probably be used as vaccine targets (Reviewed in Mullen *et al.,* 2006). To determine the invasion mechanism of *P. falciparum*, a phage display library of a random dodecapeptides fused to the N-

terminus of the phage coat protein VIII was injected into the *Anopheles* mosquito. Organs of interest were dissected and bound phage eluted. From the eluted phage, a 12- residue peptide was identified as salivary gland and midgut peptide 1 (SM1). This peptide hindered the *Plasmodium* entry into the salivary gland and the midgut epithelia, thus preventing the development of the *Anopheles* infection (Reviewed in Mullen *et al.,* 2006).

Rao and coworkers used a phage library that was displaying 12-mer random peptides fused to the PIII coat protein of M13 phage to identify peptides that could bind to the cell surface of pathogens and have bactericidal activity (Rao *et al.,* 2013a; Rao *et al.,* 2013b). They used subtractive panning phage display approach, which involved panning to remove phage that bound to *S. aureus*, prior to panning to select for phage specific to *E. coli* (Rao *et al.,* 2013a) or panning to remove phage that bound to *E. coli* and *S. epidermidis*, prior to panning to select for phage specific to *S. aureus* (Rao *et al.,* 2013b). After six successful rounds of biopanning, phage DNA was isolated for nucleotide sequencing.

Five *E. coli* specific clones (Rao *et al.,* 2013a) encoded the same peptide sequence RLLFRKIRRLKR (EC5). This peptide was found to bind specifically to *E. coli* and *P. aeruginosa*. They further investigated if this peptide (EC5) could have antimicrobial activity *in vitro*. They inoculated mid-log phase bacteria (105 CFU/ml) with 0 to 50 μg/ml of peptide EC5 and the sample was incubated in a shaker for 2 hours. The sample was plated on nutrient agar plates and the CFU was recorded. There were no colonies observed on plates inoculated with *E. coli* or *P. aeruginosa* incubated with peptide

concentrations of 12.5, 25 and 50 µg/mL. Hence, the EC5 peptide exhibited bactericidal activity against *E. coli* and *P. aeruginosa*. However, this peptide could neither bind to nor exhibit bacterial activity against gram positive bacteria like *S. aureus*, *S. epidermidis* and *B. cereus*.

The panning process used to produce *S. aureus* specific clones (Rao *et al.,* 2013b) resulted in 9 of 18 isolated clones having the same peptide sequence VPHNPGLISLQG, which was named as SA5-1. To determine the binding potential and specificity, the SA5-1 peptide was tested against a wide variety of other bacteria (*S. aureus, S. epidermidis, B. cereus, E. coli, P. aeruginosa and K. pneumonia).* Bacteria were grown to log phase, serially diluted in PBS, and spotted on a nitrocellulose membrane. The biotinylated peptide was then incubated with the bacteria on the membrane, followed by streptavidin-HRP, and the color indicating bound peptides developed using diaminobenzine (DAB). They found that SA5-1 peptide bound specifically to *S. aureus* and did not cross react with other bacteria. To confirm this test, they did an ELISA, where they found that the peptide was highly specific for *S. aureus*. In this article, the peptide was tested for its use as a diagnostic agent for *S. aureus* infections, but was not tested for bactericidal activity.

4.2 Future work.

In our study we hoped to identify a peptide that could bind specifically to S. aureus type 5 and 8. We propose that the identification of phage peptides specific for S. aureus using phage display technology could provide a promising therapeutic agent that could eradicate the pathogen.

Maratani isolated 5 phage clones specific for *S. aureus*, type 5 and Lenkey isolated 8 clones that were specific for *S. aureus*, type 8 (Lenkey 2016, Maratani 2017). Lenkey's ELISAs suffered from high background as seen in Figure 1 of these studies. In future studies, these clones will be tested again using the new block and trypsin treated ligand. In addition, the pooled capsular polysaccharides (both type 5 and type 8 from previous studies) will be used to determine if any of the previously selected clones will specifically bind to capsule polysaccharide type 5 or type 8.

If a specific clone is found, the DNA will be sequenced and pure peptide synthesized. The peptide will be tested for specificity, as well. If a specific peptide is found, it will be tested for direct cytotoxicity to S. aureus. If it is not cytotoxic, future studies will investigate the possibility of coupling a toxin to the peptide to target the toxin to *S. aureus*.

CHAPTER 5.

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