Using Phage Display to Select Peptides Binding to Type 5 capsular polysaccharide of *Staphylococcus aureus*

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ABSTRACT

S. *aureus* is a major pathogen in humans producing infections that are difficult to treat due to antibiotic resistance. This disease has mortality rate of 20 – 40% within 30 days of infection, despite appropriate treatment. Being both a pathogen and a commensal bacteria colonizing the nasal nares and other human body parts, the infection is easily spread, especially through the hands, whenever hygiene is compromised. The goal of this project is to select peptides with binding specificity to *S. aureus* capsular polysaccharide, type 5, which in the future may be coupled to an antibacterial compound and targeted against the bacteria. Utilizing phage display technology, phage have been selected from the PhD-7 phage library by biopanning and tested for binding specificity by phage ELISA. Five out of the ten clones picked following panning 3 have demonstrated strong binding to *S. aureus,* type 5, whole cells, when compared to their binding to *E. coli* bacteria, *S. epidermidis* and *S. aureus,* type 8. DNA sequencing will be done on the clones that show good binding characteristics when tested by carbohydrate ELISA.

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LIST OF ABBREVIATIONS

APC- Antigen presenting cell

MAC- Membrane attack complex

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

CHO- Carbohydrates

CTL cells- Cytotoxic T lymphocyte cells

ClfB - clumping factor B

ELISA – Enzyme linked immunosorbent assay

HA-MRSA – Hospital associated methicillin resistant *Staphylococcus aureus*

HRP – Horseradish peroxidase

HAT - Hypoxanthine Aminopterin Thymidine

IPTG - Isopropyl β-D-1-thiogalactopyranoside

IsdA, – Iron- regulated surface determinant A

mAb – Monoclonal antibodies

MHC – Major histocompatibility complex

MM SAT5- Martin Maratani *Staphylococcus aureus* type 5

MRSA- Methicillin resistant *staphylococcus aureus*

NO SA – No *S. aureus*

OD- Optical density

PAMPs- Pathogen associated molecular patterns

PBS- Phosphate-buffered saline

PFU – Plaque forming unit

PRRs – Pattern recognition receptors

scFv –Single chain fragment variable

SpA – *Staphylococcus aureus* protein A

SQS 1 - *Staphylococcus aureus* quorum sensing protein

TLRs – Toll-like receptors

VISA - Vancomycin insensitive *Staphylococcus aureus*

VRSA – Vancomycin resistant *Staphylococcus aureus*

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I. INTRODUCTION

a) *Staphylococcus aureus* Infections

Staphylococcus aureus is a gram positive bacteria known to cause a variety of infections, ranging from superficial skin infections to life threatening invasive infections such as endocarditis, meningitis, post-operative wound infections, septicemia, ventilator-associated pneumonia, septicemia associated with catheters, surgical sites infections and toxic shock syndrome (Yang *et al.,*2005 and Eko *et al*.,2015).

This bacterium is reported to be one of the main causes of bloodstream infections, with a mortality rate of 20% -40% within 30 days of infection. This is despite appropriate treatment (Brown *et al*., 2015 and Lehar *et al*., 2015). It is a major cause of hospital-acquired methicillin resistant (HA.MRSA) infections in Europe and a significant cause of community acquired methicillin resistant (CA.MRSA) infections globally. The bacteria also causes of infections in solid organ transplant tissues. (Lehar *et al.,* 2015 and Cheung *et al.,* 2016)

The symptoms of the bacterial infections are largely due to the production of exotoxins during bacterial proliferation. This process is regulated by a gene complex that is made up of SQS 1 and SQS 2. SQS stands for *Staphylococcus aureus* quorum sensing protein. SQS 1 contains RAP and TRAP. RAP is RNA III activating protein, which acts on TRAP to cause activation of the *Agr* gene to produce Arg A, B, C and D. Agr D phosphorylates Agr C, leading to production of RNA III which up regulates production of *S. aureus* exotoxins (Balaban *et al.,* 2001).

S. aureus is not just a pathogen; it is also a commensal bacterium. Thirty to forty percent of healthy people are estimated to be colonized by the bacteria in the nasal vestibule and the skin. Other anatomical sites of human body that may be colonized by the bacterium are the gastrointestinal tract and the human throat. Gastrointestinal tract colonization is asymptomatic, very hard to eradicate and is often the source of bacteria that cause soft tissue and skin infections. (Lehar *et al*., 2015 and Misawa *et al.,* 2015).

Many authors are in agreement that bacterial colonization is an important factor contributing to the bacteria infection, but disagree on which factors enhance colonization. While some argue that bacterial teichoic acid, capsular polysaccharide (type 5 and type 8) and surface proteins are factors that promote colonization (Misawa *et al*., 2015), others attribute colonization to an ineffective immune response (Reviewed in Foster, 2009). The latter is of the view that polymorphism of glucocorticoid receptor is the reason as to why some individuals are carriers, while others are not. Foster cites individuals with glucocorticoid receptor haploid 3, who usually have associated immune enhancement, stating that they have a 68% lower carriage rate.

b) ***S. aureus*** **Pathogenicity and Immune Evasive Mechanisms in Human**

S. aureus is a commensal organism residing on human skin and nares. Infections easily occur whenever there are breaches in local protective barriers as a result of skin abrasion, surgical wounds or implantation of medical devices. The host's immune system reacts to this bacterial invasion by activating innate immune mechanisms, including cytokine production which activates B and T cells and chemokine production that attracts macrophages, neutrophils and other immune cells to the site of infection. The

macrophages and neutrophils are phagocytes whose immune effector role is to ingest and destroy the bacteria. T helper cells produce cytokines that activate cytotoxic T lymphocytes to lyse the cells harboring intracellular pathogens. Although *S. aureus* bacteria can invade and survive in immune cells, including in macrophages, neutrophils and other non-phagocytic cells for a considerable period of time (McGonigle *et al* 2016), there is no clear data to show that these cells are destroyed by CTL cells. B cells are also activated by the cytokines produced by T helper cells to differentiate and proliferate into plasma cells secreting antigen specific antibodies (Zhuo *et al*., 2009). Both the effecter T cells and plasma cells may take up residence in the bone tissue as memory cells for an extended period of time after the infection has been cleared. Antibodies and complement molecules serve as opsonins coating the bacteria to facilitate phagocytosis. This process is referred to as opsonophagocytosis. Antibodies may also serve to neutralize the secreted toxins and to activate the classical complement activation pathway, resulting in the formation of the membrane attack complex, which helps to destroy the invading bacteria (Ziegler *et al*., 2011 and Owen *et al.*, 2013).

In view of this robust human immune defense mechanism, the survival of the invading bacteria in the host's body will entirely depend on the bacteria's ability to up regulate its virulence factors. The *S. aureus* bacterium uses multiple virulence factors to attach to host tissues, invade the host's body and evade the immune system producing infection even in immunocompetent hosts. Five surface *S. aureus* proteins, which include clumping factor B (ClfB), Iron –regulated surface determinant B (IsdA), SdrC, SdrD and SasG proteins, promote the adhesion of bacteria to epithelial cells of the nasal vestibule. To survive on the skin, which is acidic in nature, the MRSA bacteria utilize an

arginine deaminase enzyme to degrade arginine into ATP and ammonia, which neutralizes the acidic pH of the skin (Reviewed in Foster 2009).

To invade the host, the bacterium disrupts the barrier by secreting exfoliative toxins. These toxins cause blistering of the skin, allowing passage of the bacteria to the subdermal tissues. Bacteria invasion is triggered when the immune system is compromised and when there is breach or cut into the skin tissue. Once the bacteria have entered the host's body, it secretes various anti-opsonizing proteins that help the bacteria to evade the immune response. These proteins may include chemotaxis inhibitory proteins, which prevents migration of neutrophils into the tissues and penton-valentine leucocidin which lyses the leukocytes. Chemotactic Inhibitory Protein binds to chemo attractant peptide receptors and prevents neutrophils migration towards the sites of infection. Protein A secreted by the bacteria and found in the cell wall, modulates B cell immune functions by non – immunologically binding to Fc portion of the antibodies. It thus impairs opsonization by the antibodies. It is capable of binding non-immunologically to IgG, IgA and IgM, enabling the bacteria to escape phagocytosis. It also thought to cross-link the V_H3 B cell receptor type, resulting in superantigen-like B cell proliferation. (Thammavongsa *et al,* 2015)

By using these proteins the bacteria is able to impair the inflammatory response, resulting in decreased immune response to the bacteria, and in the process the bacteria multiply and spread to other parts of the body producing additional infections. (Reviewed in Foster 2009 and Falungi *et al*., 2013)

Capsule and biofilm formation are other mechanisms that the bacteria uses to evade the host's immune response. Studies have revealed that capsular polysaccharide

plays an anti-phagocytic role by decreasing the amount of deposition of C3 fragments on the surface of the bacteria (Lee *et al*., 2004 and Nanra *et al*, 2012). The capsule masks the surface bound C3 fragment, impairing complement receptor mediated recognition by the neutrophil. Biofilm matrix provides protection by restricting penetration of some antibiotics. It also provides protection against immune cells. (Lee *et al*., 2004)

Extracellular fibrinogen binding protein is another very important microbial immunomodulatory molecule inhibiting complement activation. The complement system is an innate immune defense that is made up of a group of more than 20 serum proteins which are activated in a cascade. The proteins ultimately form a membrane attack complex effector molecule that destroys some invading pathogenic microorganisms. Another important effect of complement activation is that it causes inflammation and chemotaxis of white blood cells to the infected site. A study carried out by Lee and colleagues showed that the extracellular fibrinogen binding protein inhibits complement activation and complement mediated opsonization by binding to C3 and preventing it from binding to its activator surfaces. C3 is a vital component in the complement cascade. When activated it forms the opsonin fragment C3b and provides a link between the alternative, classical and lectin complement pathways. All of the three pathways lead to inflammation and to the formation of the membrane attack complex molecule. The activation of C3 is required for both the classical and alternative complement activation pathways. People having a genetic C3 deficiency are highly susceptible to bacterial infections. (Lee *et al.,* 2004)

S. aureus is also capable of invading and surviving for a prolonged period of time inside various phagocytic and non–phagocytic cells. It is therefore believed that the rapid

spread of the bacterium to various parts of the body is facilitated by these infected cells serving as vehicle for the bacterium (McMagonicle *et al*., 2016). Studies also show that persistent infections with *S. aureus* and prolonged stimulation of T cell by *S. aureus* super antigens, including toxic shock syndrome toxin (TSST), results in T cell anergy. This implies that T cell is rendered non- responsive to any further stimulation (Ziegler *et al.,* 2011).

***c) S. aureus* Antibiotic Resistance**

S. aureus is capable of evolving into different strains with resistance to various commonly used antibiotics. The excessive use and abuse of antibiotics has led to the emergence of multiple drug resistant strains of *S. aureus*. Since antibiotics resistance is often encoded by the genes carried on a plasmid, resistance spreads between bacteria at a rapid and alarming rate. During the 1940s penicillin was the most effective drug treating all *S. aureus* infections. By late 1940s there was an emergence of *S. aureus* strains with a penicillinase enzyme conferring resistance to penicillin. Following this, methicillin, a drug resistant to the penicillinase enzyme, was introduced. The effectiveness of this antibiotic only lasted for a short period before the emergence of a Methicillin Resistant *S .aureus* strain (MRSA). *S. aureus* strains producing Class A beta–lactamases show antibiotic resistance to penicillin, by opening the penicillin beta–lactam ring (Reviewed in Smith and Jarvis, 1999).

This *S. aureus* strain has spread worldwide as nosocomial pathogens causing a potential public health threat, because of its resistance to multiple antibiotics drugs (Harris *et al.,* 2002).

The β-lactam antibiotic inhibits the *S. aureus* cell wall formation by binding to and inactivating penicillin binding proteins (PBP). Penicillin binding protein (PBP) is one of cytoplasmic membrane *S. aureus* enzyme that helps in assembling the murein monomers into peptidoglycan during Bactria cell wall formation. The PBP is therefore what is targeted by Methicillin antibiotic active ingredients. The other name for PBP is transpeptidase enzyme. This enzyme links the newly formed nascent peptidoglycan to pre-existing peptidoglycan layer of the *S. aureus* bacteria cell wall. Inactivating this enzyme impairs cell wall synthesis leading to bacterial cell death by autolysis due to high cell osmotic pressure. Resistance to methicillin antibiotic is conferred by acquisition of the *mecA* gene, which codes for the variant of PBP with extremely low affinity for the β-lactam molecule. These variants include PBP2a and PBP'. The *mecA* gene is highly conserved among MRSA and *Staphylococci epidermidis* Bacteria. The gene is contained in a mobile genetic element referred to as *Staphylococci* cassette chromosome (SCC *mec*). To express Methicillin antibiotics resistance, this gene is aided by a group of other genes called *factor essential for methicillin resistance.* (Braves *et al.,* 2015, Reviewed in www.cfsph.iastate.edu 2011).

MRSA may also show heteroresistance to beta- lactams. This concept implies that a single colony on subculture may produce colonies of varying antibiotic resistance. It follows therefore, that while some colonies among the population may be susceptible to a given beta–lactam antibiotic on the laboratory test, others are not. If the patient is treated with beta-lactam antibiotics, the resistant subpopulation will quickly grow and replace the susceptible sub population (Smith and Jarvis 1999).

With the evolution of bacteria strains resistant to methicillin and other antibiotics belonging to the same class as methicillin, vancomycin remained the only option for treating infections caused by the MRSA bacteria strain. This however only lasted until 1996, when the first MRSA bacteria strain showing intermediate resistance to vancomycin was reported in Japan (Breves *et al*., 2015). The distinction between the VRSA and VISA is determined by the antibiotic minimal inhibitory concentration to vancomycin antibiotic drug. It is also believed that the VISA spontaneously generates VRSA Bacteria within the cell population following continued exposure to vancomycin antibiotic drug (Braves *et al.,* 2015).

Unfortunately, many vancomycin intermediate *S. aureus* strains have been isolated in the USA, France, Korea, Scotland, South Africa and Brazil. This strain is frequently generated from MRSA bacteria strains upon exposure to vancomycin. By 2002, the first vancomycin resistant *S aureus* strain was isolated in the USA. This strain was shown to carry the *van* gene which is thought to have been acquired via genetic exchange of material from vancomycin resistant *Enterococci*. For this reason, *S.aureus* remains a very important pathogen and a center of focus for many scientific researchers. As the worldwide spread of the vancomycin resistant stain is inevitable, there is an urgent need for the discovery of a novel therapeutic intervention (Harris *et al*., 2002).

c) The Spread of Methicillin Resistant *Staphylococcusi aureus* (MRSA)

MRSA is a leading cause of multi-drug resistant nosocomial infections in the U.S. and is the strain responsible for ventilator associated pneumonia, septicemia associated with catheters and surgical site infections (Cooke and Brown, 2010; Eko *et al*.,2015). Although the cases of invasive MRSA infections in the U.S and Europe have reduced in

the past recent years, MRSA remains a public health risk. The proportion of MRSA isolates is still above 25% in 8 out of 28 countries of many Southern and Eastern Europe including Italy (Gesualdo *et al.,* 2014).

Studies show that the main mechanism of MRSA spread in a hospital setting is via the hands and therefore good hand sanitation for the healthcare worker is an important infection control measure. Although the main reservoir of the bacterium may be patients admitted with various bacterial infections, there is data to show that bacterial colonization among the individuals, including the health care workers, is a significant factor associated with the transmission of the bacterial infections in a hospital setting. (Braves *et al.,* 2015)

MRSA can either be Community Associated (CA-MRSA) or Hospital Associated (HA-MRSA) depending on the infection etiology. CA-MRSA was first recognized in mid-1990 and it was noted to be a strain of MRSA causing infections in patients in the community or entering a hospital for the first time. Several studies however, indicate that CA-MRSA is starting to infiltrate hospital settings and is causing infections with an onset of less than 72 hours after admission to the hospital. There are two main clones of CA-MRSA that have caused infection over a wide geographical area. These clones are USA 300 and USA 400. Both are believed to harbor the Panton–Valentine leukocidin (PVL) toxin genes, which encode a beta pore forming toxin. (Cooke and Brown 2010)

MRSA epidemiology varies globally and CA-MRSA is spreading very rapidly to the hospital settings. CA-MRSA and HA-MRSA are not only distinct “genotypically and phenotypically” but their differences are also noticeable at the molecular level. Unlike HA-MRSA, the CA-MRSA isolates carries SSC *mec* type IV or type V cassette, and have different exotoxin gene profiles. For example, the PVL genes that confer virulence and

transmissibility are found only in the CA-MRSA. While HA-MRSA isolates are resistant to multiple antibiotics, CA-MRSA isolates are only resistant to β lactams, macrolides, and azalides. CA-MRSA is susceptible to Vancomycin TMP-SMX, gentamycin, tetracycline, and clindamycin (Cheung *et al.*, 2016).

The HA-MRSA strains have increased morbidity and mortality rates, and lengthen the period of hospital stays, resulting in inflated healthcare costs. However, CA-MRSA is more virulent and transmissible than HA-MRSA. It is argued that CA-MRSA infections arise in otherwise healthy individuals, unlike HA-MRSA, where infection follows exposure to predisposing factors, such as surgery or the presence of indwelling medical devises (Reviewed in Tokajian,2014).

d)* Vancomycin resistant *S. aureus

Vancomycin resistant *S. aureus* (VRSA) are the MRSA bacteria resistant to vancomycin antibiotic drugs. The emergence of this strain of *S. aureus* bacteria is due to increased use and abuse of vancomycin antibiotic drugs. The emergence of vancomycin resistance in *S. aureus* Bacterial has become a global issue and a potential public health disaster. A study carried out in Brazil revealed that due to high *S. aureus* infection prevalence, there was an increased use of the vancomycin antibiotic drug. This increase in usage, together with poor sanitary conditions of some public health institutions are factors thought to have led to the emergence of vancomycin Intermediate susceptibility *S. aureus* strain (VISA), also referred to as hetero-VRSA.

In Brazil, vancomycin resistant strains were isolated following a study carried out among 140 hospitalized patients. In the USA, in the city of Michigan, the first VRSA was isolated in 2002. This strain contained *vanA* gene suggesting a possible genetic transfer of

material from *Enterococcus spp. The* VRSA strains isolated from Brazil however lacked the *vanA, vanB* and *vanC* genes upon testing using polymerase chain reaction. (Braves *et al.,* 2015, Daini and Akano, 2009)

e) Current status on *S. aureus* Vaccine and Therapeutic Monoclonal Antibody Development

With most MRSA are also multidrug resistant, and with the emergence of VRSA, the threat to revert to a pre–antibiotic error characterized by inability to treat staph infections is a very real possibility. Efforts geared towards discovery of a novel *S. aureus* vaccine and therapeutic antibodies are in progress, but as it stands now, no breakthrough has yet been reported.

i) *S. aureus* Vaccine

A vaccine is noninfectious material containing antigenic part of a given pathogen. The aim of the vaccine is to stimulate an antigen specific immune response and provide an individual with immunological memory cells against the pathogen. The importance of this is that the individual develops the immunity against the pathogen without having to suffer from the disease it causes. The vaccine material can either be a live attenuated pathogenic microorganism (attenuated *S. aureus* in this case), killed or inactivated bacteria, a toxoid (inactivated exotoxin), a subunit vaccine, a conjugate vaccine, a DNA or recombinant vector vaccine. In conjugate vaccine preparation, a highly immunogenic protein is fused to a weak vaccine immunogen or mixed with extraneous protein to enhance or supplement immunity to the pathogen (Owen *et al.*, 2013). Multivalent subunit vaccines are made by mixing an antigenic macromolecule extracted from a

pathogen with protein micelles, liposomes or immunostimulating complexes which can be mixed with detergent – extracted antigens or antigenic peptides. Recombinant vector vaccines on the other hand are made by inserting genes encoding specific antigenic determinant into a plasmid vector, which serves to multiply and display the antigen for immunological recognition by immune effecter cells. DNA vaccines also utilize plasmid DNA encoding the antigenic protein, except that in this vaccine strategy the plasmid is injected directly into the muscle of the recipient with the hope that host cells will take up the DNA and produce the immunogenic protein in vivo. (Owen *et al.*, 2013)

Vaccination is therefore a form of active immunization meant to induce the body to produce antigen specific immune effector cells and molecules. Some of the antigen sensitized and differentiated immune effecter cells of the adaptive arm of the immune system (T lymphocytes and B lymphocytes) will cross over from the peripheral circulation and enter the tissues were they may take residence for a long period of time as memory cells. These memory cells are always on guard and in an event that an infection occurs, they respond more rapidly and strongly to clear the invading pathogen. (Owen *et al.*, 2013)

Vaccines also have multifaceted effects on the cells of the innate immune system. Very important among these cells are the macrophages and the myeloid dendritic cells. These two cells, together with to B cells, serve as professional antigen presenting cells (APC) in addition to having other immune effecter roles. The APC have several types of pattern recognition receptors (PRRs) that serve to recognize evolutionarily conserved antigenic parts of bacteria (Owen *et al.*, 2013). The conserved pathogen patterns are collectively referred to as pathogen associated molecular patterns (PAMPs). The vaccines

are therefore engineered to contain these PAMPs. Upon the introduction of the vaccine into the body, the presence of the PAMPs is detected via the PRRs which induce signal transduction, leading to APC activation. APC activation results in up regulation of costimulatory molecules and the expression of pro-inflammatory cytokines. The vaccine activated APC would therefore be able to ingest, process and present a fragment of the antigen on major histocompatibility complex (MHC) I or II. This way, the APC activates CD8+ and CD4+ T cells, respectively, (Zhuo *et al*., 2008) note that CD4+ T cell have Toll like receptors, TLRs, (PRRs) and may as well be stimulated directly on contacting the receptor specific ligands.

The CD4+ T cells and the activated macrophages also produce cytokines that promote B cell growth and activate other cells, including monocytes and Natural Killer (NK) cells. This immune cell activation process culminates in differentiation and proliferation of the B cells into plasma cells, which secrete antigen specific antibodies, and B memory cells for future protective actions. It also results in the activation of phagocytic cells that clear the invading bacteria via opsonophagocytosis. The antibodies may also coat the pathogen making it harmless. This process is referred to as neutralization. The antibodies may also eliminate the invading pathogen via classical compliment activation.

Unfortunately, S. *aureus* bacteria have developed mechanisms to invade the host's body, evade the immune response mounted by the host and produce infection, despite the presence of activated immune cells and antibodies. As a result, CA-MRSA is reported to cause infections in otherwise healthy and immune competent individuals (Zhou *et al*., 2008, Owen *et al.*, 2013 and Lee *et al*., 2004).

S. aureus vaccine may be engineered to contain antigenic macro molecules such as peptidoglycan or lipoteichoic acid. The vaccine may then be targeted to TLR2 on the myeloid dendritic cell. Upon recognition of these bacteria components, PAMPs, by the TLR 2, the immune system would be activated. Novel vaccine development for *S. aureus* is however more complicated than it hypothetically appears. Indeed, various vaccine strategies have been tried with no success. The difficulty is thought to be partly due to the bacteria virulence factors that dampen both the innate and the adaptive immune responses. Since many attempts that appeared to work in murine models have never translated to protection in human, there is also a school of thought that the murine model may not be fully representative of human immune system. This is further complicated by the fact that *S. aureus*, being a commensal microorganism, has over time adapted to survive under the hostile environmental conditions created by the human immune response (Reviewed in Tokajian 2014, Smith and Jarvis 1999 and Brown *et al.,* 2015).

ii) **Therapeutic Monoclonal and Polyclonal Antibodies Against *S. aureus* Bacteria**

Monoclonal antibodies are antibodies produced by a cell descending from a single clone. Usually monoclonal antibodies are produced by cloning hybridomas. A B cell from an immunized mouse is fused to a myeloma cell to produce a hybridoma. The hybridoma cell is immortal and produces antibodies in vitro indefinitely. The antibodies are collected from the culture media and screened for binding to a pathogen (Pandey, 2010).

On the other hand, polyclonal antibodies are antibodies produced by different clones of B cells. They are usually antibodies collected from the sera of an animal with prior exposure to antigenic material (Pandey, 2010).

Some of the immune effecter functions of the antibodies include coating the invading Bacteria (pathogen) to facilitate phagocytosis. This effecter function is referred to as opsonization. The antibodies may also coat and renter harmless the pathogen or molecules secreted by the pathogen. This process is known as neutralization. The antibodies can also initiate complement activation, which ultimately results in inflammation and the formation of a membrane attack complex that can kill invading bacteria (Owen *et al.*, 2013).

The first monoclonal antibodies produced were mouse antibodies (Kholer and Milstein, 1975). These antibodies have been used to treat many human diseases, however their use is limited because of the immune response to the antibody and the side effects associated with it. Human mouse chimeric antibody followed, then humanized antibodies and, subsequently, fully human antibodies. Fully human antibodies can either be prepared using transgenic mice or phage display with scFv antibodies (Nian *et al.,* 2016)

Previously, our lab was involved in the production of monoclonal antibodies against *S. aureus* type 5 and 8 capsular polysaccharides (Fagan *et al* 2004). Splenocytes were harvested from a mouse immunized with *S. aureus,* type 5 and 8, formalinized and trypsinized whole bacteria, fused with myeloma cells and cloned by a limiting dilution method. Hypoxanthine, Aminopterin, and Thymidine (HAT) were used to select for fused cells. The myeloma cells provided genes for continued cell division in tissue culture while the splenocytes provided the functional immunoglobulin genes for antibody

production and the genes for resistance to HAT. The cells were fed and maintained in the tissue culture and the antibodies harvested and tested for specificity against *S. aureus* antigen using ELISA (Pandey, 2010).

For an extended period, this appeared to be a possible therapy for Staphylococcus infections, but many studies have shown that the antibodies simply do not work. (Reviewed in Tokajian 2014, Smith and Jarvis 1999 and Brown *et al.,* 2015). This implies that vaccines would equally not work since the ultimate vaccine effector mechanism is premised on antibody production and immune phagocytic cells activation. Of course as earlier mentioned, there is evidence that the bacteria may indeed survive the degradative action of bioactive molecules in the phagosome of phagocytic cells (Thammavongsa *et al.,* 2015). By extension, the antibodies produced in vitro and artificially administered are confronted with the same fate.

We have seen numerous studies showing how *S. aureus* infection take root and disseminate in the body, despite the presence of high titers of antibodies against the bacteria. Studies now show that the vaccines and therapeutic antibodies that appear to work in animal models have always failed at the clinical level when tested for efficacy in humans (reviewed in Fowler and Procter, 2014). Just as the bacterium uses its virulence factors to invade the human body, evade and dampen both the innate and the adaptive immune response to produce infection in an immunocompetent individual, so does it avoid the vaccine induced immune response and the therapeutic antibodies administered.

However in contrast, some studies have shown that a subunit vaccine made, which included SpA, generated protein A neutralizing antibodies in mice and the antibodies protected the vaccinated animal against S. *aureus* abscess formation. It is also

noted that the neutralizing antibodies helped the *S. aureus* infected animal mount an effective humoral response against different bacterial antigens (Falungi *et al.,* 2013). It is based on this observation that Falungi and colleagues did a study to investigate the role played by the SpA in immune response modulation. It was discovered that SpA has a five-immunoglobulin binding domain protein anchored in the bacteria membrane and it is released during bacterial growth. This is how the bacterium modulated the immune response.

Like SpA, *Staphylococcus aureus* extracellular fibrinogen binding protein (Efb) has been implicated as a cause for failure of vaccine and antibodies against *S. aureus* bacteria. Efb is a 16 kD protein found in 85% of *S. aureus* strains that has two functional domains with two binding sites for fibrinogen and complement. By binding to both fibrinogen and complement, the Efb protein generates a capsule- like shield around the bacteria surface, masking important opsonic molecules, like C3 and antibodies, from binding to phagocyte receptors. Through this process, the bacteria can block the process of phagocytosis (Ko *et al.,* 2013).

The ability of the bacterium to withstand degradation and remain viable for long inside the phagocytes and other non-phagocytic cells undermines the innate immune response. The bacterium is also said to use these infected cells to spread to other parts of the body. Patients with a normal neutrophil count are therefore more prone to disseminated *S. aureus* infections. This infection is mostly chronic and recurrent (McGonigle*et al.,* 2016).

In contrast to other vaccine studies, Lehar and coworkers showed that an antibody-antibiotic conjugate vaccine consisting of anti-*S. aureus* antibodies covalently

linked to an antibiotic via a cathepsin-cleavable linker could eradicate intracellular bacteria. However, the study was however carried out in a murine model. Since there is no record to show that the conjugate therapeutic compound has been subjected to clinical trial, the success of this compound remains speculative (Lehar *et al.,* 2015).

Nanra and colleagues in their study identified *S. aureus* capsular polysaccharide type 5 and 8 as key among the bacteria immune evasive and immune modulatory virulence factors. Nanra and colleagues therefore proposes for the inclusion capsular polysaccharide antigen in the formulation of a multi-component vaccine. Unfortunately, this vaccine was not effective in clinical trials. (Nanra *et a.,l* 2012)

f) **Phage Display Technology**

Even as the world stares into a possible return to the pre–antibiotic era, one option largely remains unexploited for MRSA and VRSA therapeutic intervention. This is the use of short therapeutic peptides. Phage displayed peptides have been used in the production of biotechnologically engineered single chain fragment antibodies (Nian *et at.,* 2016). The peptides have been used to develop bioprobes identifying markers on cell surfaces and pathogenic microorganism. Biomarkers have been utilized as targets for pharmaceutical drugs and for diagnostic detection of the pathogens and pathogenic cells in the body (Rao *et al*., 2013).

Invented in 1985, phage display has remained a very important tool in different areas of science and scientific research. Other areas where this technology has greatly been utilized include: epitope mapping, protein–peptide and protein–protein interactions, protein–DNA interactions, immunology, cancer research, and drug delivery, among others (Zhou *et al.,* 2015). It has also been utilized in selecting small peptide ligands, not

only through in vitro processes, but also ex-vivo processes. scFv antibodies are primarily prepared through this technique and currently antibodies made using phage display are at different stages of pre-clinical and clinical testing as novel therapeutic compounds (Zhou *et al.,*2015).

Phage display technology utilizes a bio-panning affinity selection method to isolate ligands that have high binding affinity to the target immobilized on a solid support material. The peptides are known to form various tertiary structures, making it easy to immobilize on any surface through physical adsorption (Rao *et al*., 2013).

The principle underlying phage display technology is coupling a peptide, antibodies, or protein on a gene coding the phage coat protein, so that the protein may be displayed on the surface of the phage. Through a bio panning process, a target is used to retrieve phage clones displaying target binding peptides from a library of variants (Nielsen *et al*., 2016).

Ff Bacteriophage are viruses known to naturally infect *E. coli* bacteria bearing an F-pilus. They then hijack the bacteria machinery and use it to propagate the phage. The Ff family of the filamentous phages, consisting M13, f1 and fd, are most often used in phage display technology. These accommodate a larger DNA. They do not lyse the host bacterium, but instead export the newly synthesized phage through the cell wall. M13 phage replication is episomal, producing phage progeny up to a titer of 10^{13} pfu per mL in small scale production(Warner *et al*., 2014)..

The M13 phage is about 6.6 nm in diameter and 880 nm in length. Its length is covered by 2700 copies of the major coat protein (p VIII). One end is capped with pIII/pVI and the other end with pVII/ pIX, 3-5 copies each. The latter two pairs are minor

coat proteins. The entire genome is made up of 9 genes in total and 6, 407 nucleotides in length. The 9-gene product is however 11 proteins, including pX and pXI (Warner *et al*., 2014).

The two most often used coat proteins to display peptides are p III and p VIII. Both the proteins have important N-terminal signal sequences required for targeting the protein to the inner membrane. When displaying larger inserts or proteins, pIII is often used. The N1 and N2 domains of p III are required for phage infection to be possible. The pIII fusion is therefore commonly expressed from phagemid vectors, to avoid compromising the phage infectivity. The phagemid vector contains the plasmid, an f1 origin of replication, the f1 packaging signal, cloning restriction sites and the antibiotic resistance gene, which serves as a selection marker. The use of phagemids enhances the transformation efficiency and saves the host from the toxic effects of most pIII fusions. Hybrid phage or phagemid vectors always result in monovalent displays.

Polyvalent displays on the other hand are produced when a natural phage promoter is used in infection. This has a great bearing on the affinity of the binders. Monovalent display allows for selection and identification of strong binders purely based on affinity while the polyvalent display leads to the selection of weak binder's due to higher binding avidity (Fevre *et al*., 2014 and Ayriss *et al.,* 2006).

Once a phage display library has been constructed, screening for the variant peptides with desirable binding affinities is done through bio-panning. The ligands of interest are coated on a solid support material and the phage in solution is applied to allow the binding of specific phage variants. Non-binding phage are washed away, while the bound phages are eluted using detergents or through varying of the pH. At least three

rounds of panning are required to eliminate the non-specific binders. However, if a variety of binders are in the library, the higher avidity binders may out compete the lower affinity binders and this could present a problem, especially when dealing with cDNA library (Na-ngam *et al.*, 2008).

g) Project Goals

In this study we propose that phage display can be used to select for peptides with high binding affinity for *S. aureus*, type 5, capsular carbohydrate. The phage selected will be tested for specificity using phage ELISAs. Specific peptide sequences may be used therapeutically in future studies to target toxins to *S. aureus*.

III. Methods

A. Method for the preparation of bacteria

S. aureus bacteria was grown in Columbia broth containing 2% NaCl overnight at 37°C, with shaking at 210 rpm. Cells were pelleted by centrifuging at 13,000 X *g* at 4°C for 30 min. The cells were then vortex to a smooth suspension and resuspended in 10 ml PBS containing 1% BSA. This was done three times, with the final wash containing no BSA. The cells were treated cells with 3% Formalin overnight at 4°C. Cells were washed again with PBS containing 1% BSA or PBS 3 times and treated with Trypsin (1mg/ml) at 37°C overnight with shaking at 210 rpm. Finally cells were washed with PBS containing 1% BSA or PBS three times and brought up to optical density of 1.0 at 550 nm (or highest possible with all samples the same) (Thakker *et al.,*1998 and Sinha *et al.,* 1999).

B. Surface panning

Formalinized and trypsinized bacteria at an OD of 1 at 590 nm in PBS was added (150 μl) to each of six wells of a 96 well microtiter plate (tissue culture treated with poly-L-lysine). Block (1% casein in TBS) was added to two pre-adsorption wells. The plate was incubated at 37^{0}C overnight in a humidified container (e.g., a sealable plastic box lined with damp paper towels), followed by centrifugation at 400 x g for 15 min. Plates were stored for several weeks (depending on target stability); discarded if mold is evident on the paper towels. The procedure can stop after the blocking step or after any of the panning steps if the phage are stored a microfuge tube and the plates in the humidified chamber in the fridge. Blocking or washing solution is left on the plate to avoid drying out.

The bacteria were aspirated from all wells on day 2 and each well completely filled with 230 µl blocking buffer (1% Casein TBS in 4 wells for the first and third panning and 1% BSA in the second panning wells). The blocking solution is then aspirated from the 2 casein only pre-adsorption wells and the 2 wells were rapidly washed 6 times with PBS. Washing is done quickly to avoid drying out the plates.

Phage (2×10^{11}) in 100 µl of PBS + 0.1 % casein was pipetted into casein only pre-adsorption wells and rocked gently for 2 hours at room temperature. The phage was collected from pre-adsorption wells into microfuge tubes and stored with plates in fridge. Phage (5 µl + 5µl glycerol) was collected and stored in freezer for subsequent titers. The sample was labeled R.pa.MM (R=AmpR library, pa=preadsorption sample, MM=your intitials).

The two panning wells containing *S. aureus* and casein (panning round 1 and 3, or BSA for panning round 2) were washed 10 times with PBS. Phage was transferred from the pre-adsorption well to the first panning wells (wells blocked with casein). For panning 1, 2 and 3 the plated was rocked gently for 2 hours at room temperature and the non-binding phage discarded. The bound phage were eluted by adding 100 µl of elution buffer (500 mM KCl and 10 mM HCl, pH2) and rocking the mixture gently for 5 minutes.

The eluate was pipetted into a microcentrifuge tube, and neutralized with 25 µl of 20 M Tris-HCl, pH 8. A phage sample (5 µl + 5ul glycerol) was collected and stored in freezer for subsequent titers. This was labeled R.P1.MM. After the second panning round, the name was changed P2, and the same for P3. The phage was the titered and

amplified after each panning round. For P3 only, 125 µl sterile glycerol was added, vortexed and stored at -20°C.

For P2 and P3 the plates were saved in refrigerator while titering and amplifying phage. Specific (binding) phage were then transferred to the next panning wells and the panning steps were repeated. Unamplified P3 eluate was titered and saved by adding 50% glycerol before freezing. Plaques from this titering process were picked and amplified, then used for sequencing and for ELISAs.

Amplified phage may be stored for 5 yr or more by adding an equal volume of sterile glycerol, vortexing and storing at -20°C. This method was not used for storing phage that were to be used for panning. (Thakker *et al*., 1998and Mark *et al*., 2002*)*

C. Titering

Four LB-tetetracycline (tet) (1% Bacto-Tryptone, 0.5% yeast extract, 0.5% NaCl, containing 20 mg/ml tetracycline) plates were pre-warmed, for at least one hour, The four LB tet plates were labeled as per expected dilution (10^2, 10^4, 10^6,10^8,10^{10}) and incubated at 37°C until ready for use. Top Agar (1% Bacto-Tryptone, 0.5% yeast extract, 0.5% NaCl, 0.7% Bacto-Agar) was melted in microwave and 3 ml dispensed into sterile culture tubes, one per expected phage dilution. The media in the tube was maintained at 45°C. Xgal and IPTG (4% each) were thawed and 40 µl of each pipetted into the four tubes containing the top agar media.

Overnight bacteria culture was placed in four labeled microfuge tubes. The culture was prepared by inoculating 10 ml of LB-tet medium broth with a colony of *E. coli* (ER2738) and incubated over night at 37^{0}C on shaker at 250 rpm. Serial dilutions of

phage in LB media were made by pipetting 2 µl of phage in 198 µl of PBS in a labeled microfuge tube. Mixing was done by votexing.

Infection was performed by adding 20 µl of each phage dilution into each of the microfuge tubes containing bacteria. The tubes were vortexed and incubated at room temperature for 1-5 minutes. The infected cells were transferred, one infection at a time, to culture tubes containing 45°C warmed top agar, containing 40 µl 2% X-gal and 40 ul 2% IPTG. The microfuge tubes were vortexed briefly and immediately poured onto the pre-warmed LB-tet plates for each dilution. The plates were gently tilted and rotated to spread the top agar evenly and then allowed to cool for 5 minutes. They were then inverted and incubated overnight at 37^0C.

The plaques on the plate that had approximately 100 plaques were counted and multiplied by the number of each dilution for that plate to get the phage titer in plaque forming (pfu) units per 10 µl. After titering the eluate from panning round 3, 5 isolated plaques were picked using a pasteur pipette and bulb. The plaques were ejected into 100 µl of LB broth and stored at 4^0C. (Barbas *et al*., 2001 and Maniatis *et al.,* 1982)

D. Amplification

Cells from the LB-tet plate or from a plaque were inoculated into 20 ml of LB tet media in a 50 ml culture tube and incubated overnight at 37^oC shaking at 250 rpm. On the second day, 40 ml of Terrific Broth (1.2% Bacto tryptone, 2.4% Bacto yeast extract, 0.4% glycerol, 0.017M KH_2PO_4 and 0.072M K_2HPO_4) with Tet was inoculated with 800 µl of the overnight culture in 25 ml in an Erlenmeyer flask. The flask was then incubated at 37^oC and shaking at 250 rpm for 1 hr.

The mixture was then shaken slowly (100 rpm) at 37°C for 10 min to allow regeneration of sheared F pili before 4 x 10^9 phage particles were added to 20 ml cells (approximately 10^{10} cells in 10 ml) and mixed by swirling. Incubation at 37°C shaking at 250 rpm continued for 4-0.5 hr. The cells were removed by centrifugation at 4500 *x g* for 10 min, the supernatant was transferred to a fresh tube and centrifugation repeated.

Top 32 ml of supernatant was transferred to a new tube while saving 500 µl. 500 µl sterile glycerol was added to the saved sample and it was stored at -20°C. To the remaining solution, add 8 mL of 2.5 M NaCl/20 % PEG-8000 (w/v) was added and briefly mixed. The phage was then precipitated overnight at 4°C.

On day 3 phage were pelleted by centrifugation at 12,000 *x g* at 4°C for 15 min and the supernatant decanted. The pellet was resuspended in 2 mL TBS, transferred to 2 Eppendorf tubes and centrifuged briefly to remove any cell debris. The supernatant was then transferred to a fresh tube and 200 µL of 2.5 M NaCl/20% PEG-8000 was added and incubated on ice for 15-60 min. The tubes were then centrifuged at 12,000 -14,000 rpm in a benchtop centrifuge for 10 min and the supernatant discarded. This was followed by another brief centrifugation and the remaining supernatant was removed with pipette. The pellet was resuspended in 200 µL TBS. For long-term storage at -20°C, we added 200 µL sterile glycerol. (Barbas *et al*., 2001 and Manatis *et al., 1982*)

e) M13 Elisa

An M13 ELISA was used to test the binding specificity of selected phage clones to the *S. aureus*. A plate (96 well poly-L- lysine coated) was coated with the ligand (100 µl of formalized and trypsinized *S. aureus* bacteria in PBS), three wells for the negative control (test of M13KE without peptide), three wells for the blank (all reagents, except

phage), and three wells for the test (specific phage tested for binding to *S. aureus*). Three wells without *S. aureus* (NO SA) were coated with casein blocking buffer (1% casein in TBS) to test for the presence of phage that non-specifically bind to casein blocking buffer. (Thakker *et al*., 1998, Mark *et al*., 2002 and Sinha et al., 199*9)*

The positive control well was coated with 100 µl (2 x 10^{11} pfu) AmpR (amplified PhD-7 library) phage in 0.5 M Na_2CO_3, pH 9. The plate was incubated overnight at 37^0 C in a humidified plastic container and then centrifuged for 15 min at 1200 rpm (400 *x g*). The coating solution was then aspirated and the wells washed once using was buffer (1 X PBS), blocked using 200 µl casein blocking buffer and incubated for 30 min at 37^0C. The wells were then washed six times using 1X PBS washing buffer. (Thakker *et al*., 1998, Mark *et al*., 2002 and Sinha et al., 199*9)*

The positive control and the blank wells were then coated with 100 µl 0.1% BSA in PBS sample buffer (receiving no phage). The negative control wells were coated with 2 X 10^{11} pfu M13KE (Negative control) phage in PBS containing 0.1% BSA. The test and NO SA wells were coated with 2 X 10^{11} pfu P3 Amp (phage following the third panning round) or the specific clones to be tested (MM SAT5, clones 1-10). (Thakker *et al*., 1998, Mark *et al*., 2002 and Sinha et al., 199*9)*

The plate was then incubated for 1 hour at room temperature, washed six times using wash buffer (PBS) and then 100 µl of 1:5000 dilution of HRP/ M13 conjugate monoclonal antibodies was added to each well. After 1 hour of incubation at room temperature, all the wells were washed six times using wash buffer, then 100 µl of TMB substrate was added. The plate was incubated for up to 30 minutes at room temperature until color developed in the positive control. Stop solution (2N H_2SO_4) was added and the

absorbance read at OD 450. (Thakker *et al*., 1998, Mark *et al*., 2002 and Sinha et al., 199*9)*

Elisa values were first tested by comparing the data for the blank to the data for phage incubated with ligand using a Student's T test (Microsoft Office Excel data analysis). If the data was significantly different from the blank ($p < 0.05$), the phage incubated with ligand was compared to the phage incubated without ligand using a Student's T test. Significance was indicated if the P value was </= 0.05).

RESULTS

Bio panning was used to isolate phage displaying peptides that bind to the *S. aureus* bacteria from the PhD-7 phage library. Three rounds of bio-panning were performed and a plaque assay was used to quantify the phage yield form each panning round (Table 1). Amplification and tittering was performed on eluted phage after every panning round. The phage was incubated in wells containing casein block prior to being added to the first panning wells to remove casein specific phage. The three panning rounds then alternated blocks, with casein being used in panning rounds one and three and BSA block in panning round two. First round of bio panning yielded $2.5x10^7$ pfu/ml of specific phage. After amplification, the phage were subjecting to a second round of bio panning, there was no increase in the the phage population, resulting in a titer of $2.0x10^6$ pfu/ml, however after panning round 3, the phage number considerably increased reaching a titer of $2.8x10^{11}$ pfu/ml. Amplification of the phage eluted following panning round 3 further increased the number of phage, reaching the titers of$1.35x10^{16}$pfu/ml.

To isolate single phage clones, the phage from panning round 3 was used to infect an overnight culture of *E. coli* bacteria and serial dilutions were plated on pre-warmed LB-tet plates with top agar, X-gal and IPTG (Figure 1). The inoculated plate was incubated overnight at 37^0C and ten plaques were carefully picked and amplified (Table 2). The plaques were named MM SAT5 and numerically numbered from one to ten. Except for clones MMSAT5-3, MMSAT5-4, MMSAT5-5 and MMSAT5-6, all the clones considerably increased in the phage population after amplification.

All the clones were tested for binding specificity to *S. aureus* bacteria (Figures 2-6). Clone 1 (MMSAT5-1) appeared to bind strongly to the target in the first ELISA (Figure 2), but

Table 1: Titer for library phage before and after amplification (in pfu/ml).

Panning Round[a]	Before Amplification[b]	After Amplification
P1	$2.5x10^7$	$4.8x10^7$
P2	$2.0x10^6$	$1.2x10^5$
P3	$2.8x10^{11}$	$1.35x10^{16}$

[a]PhD-7 library was panned against *S. aureus,* type 5 to select phage that bind specifically to the bacteria. This was done by incubating the PhD-7 library with formalized and trypsinized *S. aureus* bacteria coated on a 96 well Poly-L-lysine microtiter plate that was subsequently blocked with casein. The library had been previously pre-adsorbed to casein blocking buffer to deplete phage that bind to casein. The sample buffer in which the phage was diluted was 0.1% casein in PBS. Elution buffer was made up of 500 mM KCL and 10 nM HCl, pH2. The eluted phage was neutralized with 2 M Tris HCl, pH 8. Three rounds of panning were performed with amplification between panning rounds.

[b]The phage were titered, plaques were counted and the number obtained multiplied by dilution factor to get the phage titer in plaque forming unit (pfu/ml). After panning round three, eluted phage were plated on LB-tet agar media to obtain a titer and 10 clones picked randomly and amplified. Amplification was done by infecting

overnight *E. coli* bacteria cultures with the phage. The phage particles were separated from the bacteria cells by centrifugation and purified by PEG precipitation on ice. The final phage pellet was re-suspended in PBS.

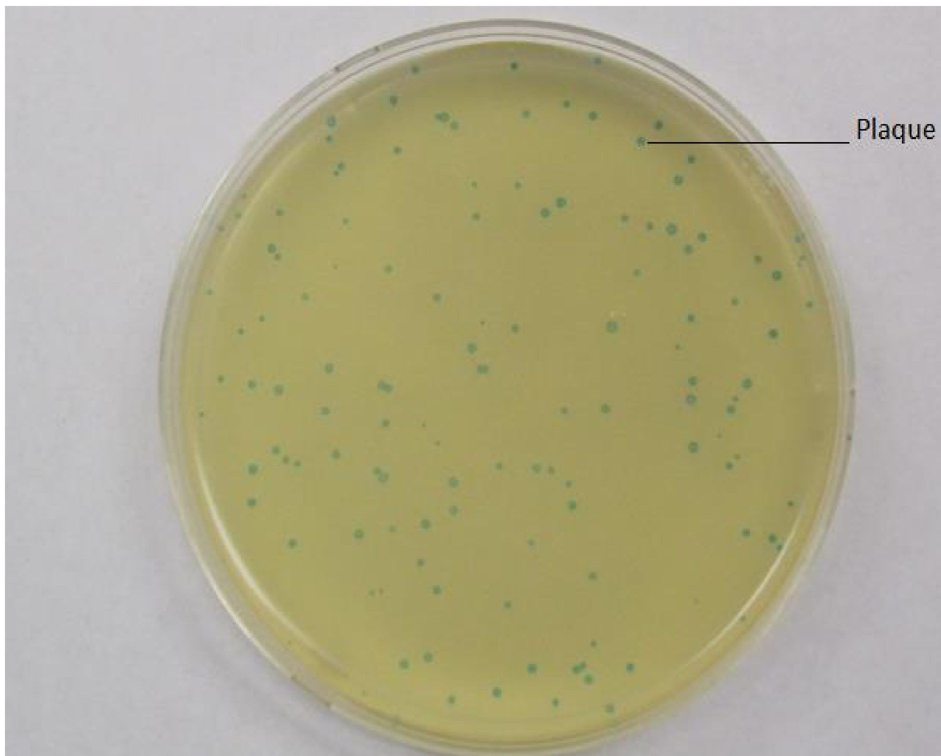

Figure 1. LB agar plate showing plaques formed during phage titration. Overnight *E. coli* bacteria culture in LB broth with Tetracycline (Tet) was infected with phage serially diluted in PBS and mixed with Top Agar, melted and maintained at 50^0C. The top agar contained 40 ul of 2% IPTG and 40 ul of 2% X-gal. The inoculum was overlaid on a pre-warmed LB Tet plate, allowed to solidify and then incubated overnight at 37^0C. Phage titers were obtained as described in Table 1 above. After bio panning round 3, ten plaques were picked and amplified, as explained in the Table 1. The plaques were named MMSAT5-1 through MMSAT5-10.

Table 2: Phage clones picked following biopanning round 3 titers.

PHAGE CLONES[a]	PHAGE TITERS BEFORE AMPLIFICATION[b]	PHAGE TITERS AFTER AMPLIFICATION
MM T5- 1	2.0×10^{13}	5.9×10^{15}
MM T5 - 2	4.55×10^{12}	5.2×10^{16}
MM T5- 3	4.0×10^{14}	4.3×10^{12}
MM T5 - 4	9.3×10^{16}	2.8×10^{14}
MM T5 - 5	1.8×10^{14}	4.3×10^{13}
MM T5 -6	8.0×10^{13}	6.5×10^{11}
MM T5 - 7	6.0×10^{12}	3.0×10^{13}
MM T5 - 8	3.5×10^{12}	2.0×10^{17}
MM T5 - 9	3.5×10^{10}	4.8×10^{13}
MM T5 - 10	9.2×10^{14}	5.7×10^{16}

[a]The table above shows the selected ten phage clones with their titers before and after amplification. Phage amplification and titration was done as described in Table 1.

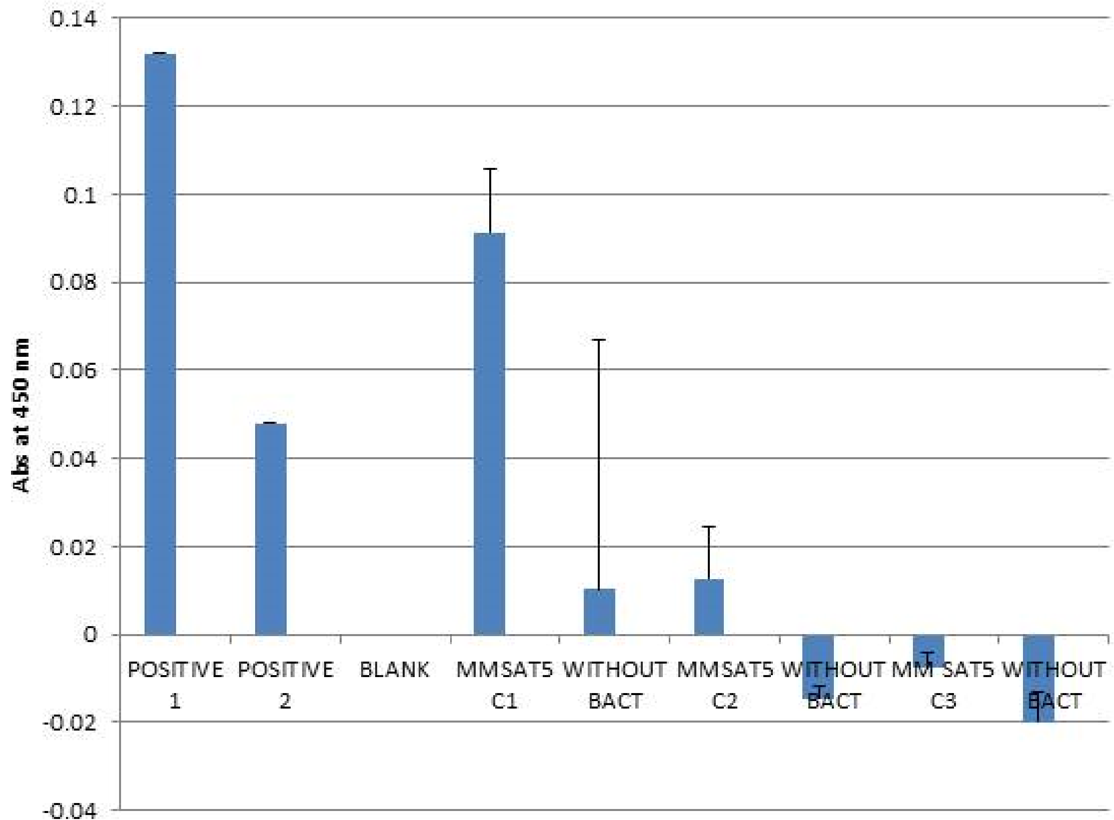

Figure 2. Phage ELISA testing of clones 1, 2, and 3 for binding to *S. aureus,* type 5. A 96 well poly-L-lysine coated plate was incubated with formalinized, trypsinized *S. aureus* for negative control wells, test wells and the blank wells. The No *S. aureus* wells were filled with casein blocking buffer. AmpR was diluted in $NaHCO_3$ for the positive control well. The plate was incubated overnight at 37^0C and centrifuged at 400 x *g*. The plate was washed with PBS and all the wells blocked using 1% casein blocking buffer. The M13KE and the phage clones were diluted in sample buffer made of 0.1% BSA 1x PBS. The phage clones being tested (2 x 10^{11} pfu) were introduced into the test and the No *S. aureus* wells wells, and M13KE into the negative control wells. Sample buffer was added into the blank wells (instead of phage). Following incubation and washing, an anti-M13 antibody, conjugated to horseradish peroxidase

was added. After incubation and wash, TMB substrate was added and the optical density was read at 450 nm as soon as a suitable color has developed. The absorbance of the blank wells was subtracted from the average OD obtained for each sample. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation.

bound with variable affinity to the no bacteria wells. However, clone 1 was shown to have significant binding to *S. aureus* in Figure 3 ($p = 0.0004$). Due to the variable results in Figure 2, Clone 1 was not pursued further. Clone 2 did not show strong binding in Figure 2, but when tested again (Figure 6) was also show to have significant binding to *S. aureus*, type 5 ($p = 0.0005$). Clone 3 was not shown to have any binding to the bacteria in Figure 2 and was not tested again. In the repeat ELISA for clone 1 (Figure 3), it was tested along with clones 4 and clone 5. Clones 4 and 5 showed near similar binding characteristics to clone 1, with significant binding ($p = 0.0023$, and 0.0017, respectively). ELISA tests for clone 6, clone 7 and clone 8 (Figures 4 and 6) showed that both clone 6 and 7 have good binding characteristics. The binding of clone 6 however was outstanding giving higher OD as compared to both positive control and clone 7 (Fig. 4). Both clones had significant binding ($p = 0.0017$, and 0.045, respectively). Clone 8 showed no demonstrable binding and was not tested again. An ELISA test performed for Clone 9 and clone 10 (Figure 5) revealed the clone 9 is a better binder than clone 10. Clone 9, however was not selected because the binding was weak and there were several other clones showing better binding characteristics. Clone 10 did not have demonstrable binding and was not tested again. The five clones out of the total of ten that had shown good binding characteristics were selected and subjected to another ELISA test (Figure 6). The clones included: MMSAT5-2, MMSAT5-4, MMSAT5-5, MMSAT5-6 and

MMSAT5-7. The ELISA test was carried out to compare the binding characteristics of the clones. All the five selected clones appeared to bind to the target (*S. aureus* type 5 cells) in a similar magnitude, as reflected by the OD obtained.

ELISAs were done with various bacteria serving as target, to test the specificity of the clones. This was meant to test whether the selected phage clones can also bind to strains of bacteria other than *S. aureus,* type 5. *S. aureus* is divided into types, based upon capsule

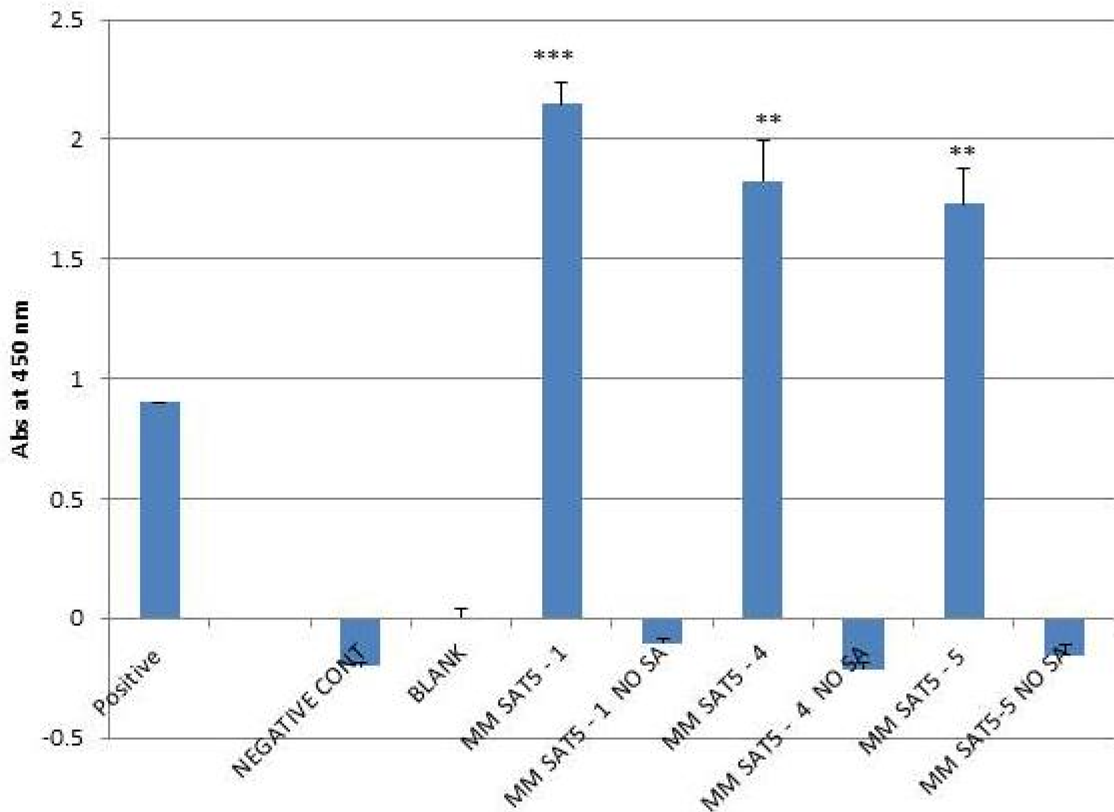

Figure 3. M13 ELISA of phage peptide clones 1, 4 and 5 for binding to *S. aureus*, type 5. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control

well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation.

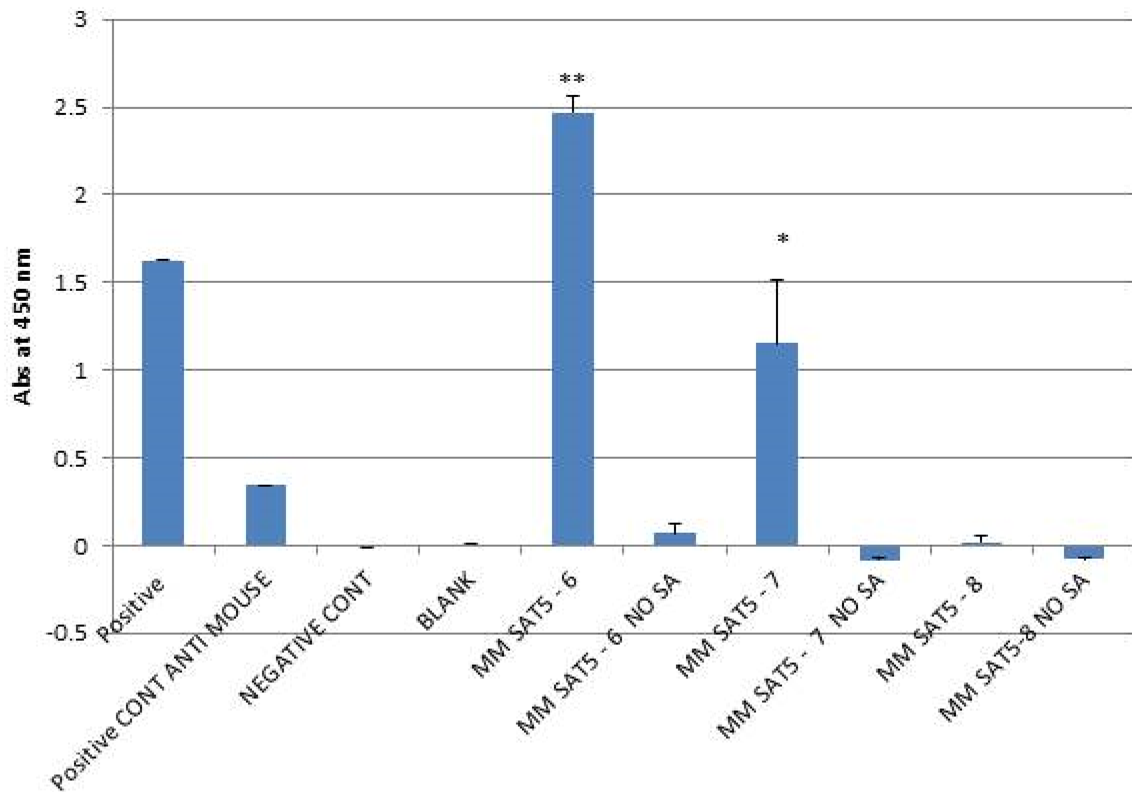

Figure 4. M13 ELISA of clones 6, 7 and 8 for binding to *S. aureus*, type 5. The ELISA was performed as described in Figure 2. Two different antibodies were used as positive control, as shown in the figure. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation.

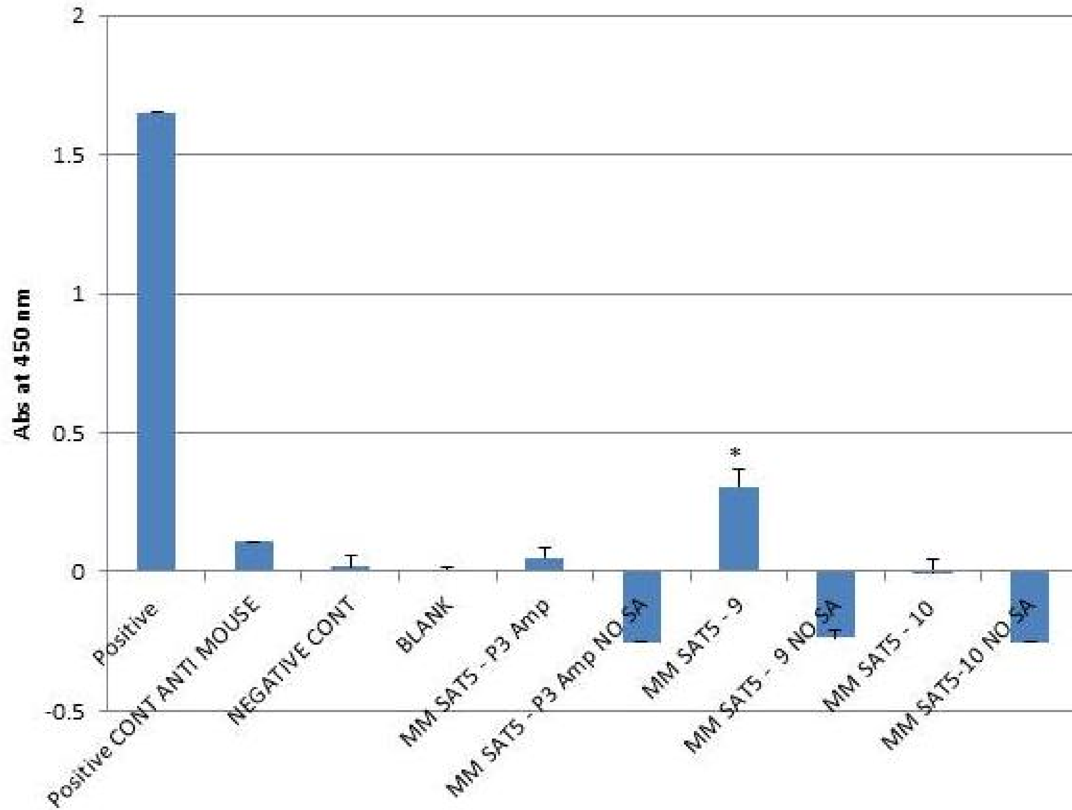

Figure 5. M13 ELISA of clones 9 and 10 for binding to *S. aureus*, type 5. The ELISA was performed as described in Figure 2. Two different antibodies were used as positive control, as shown in the figure. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation.

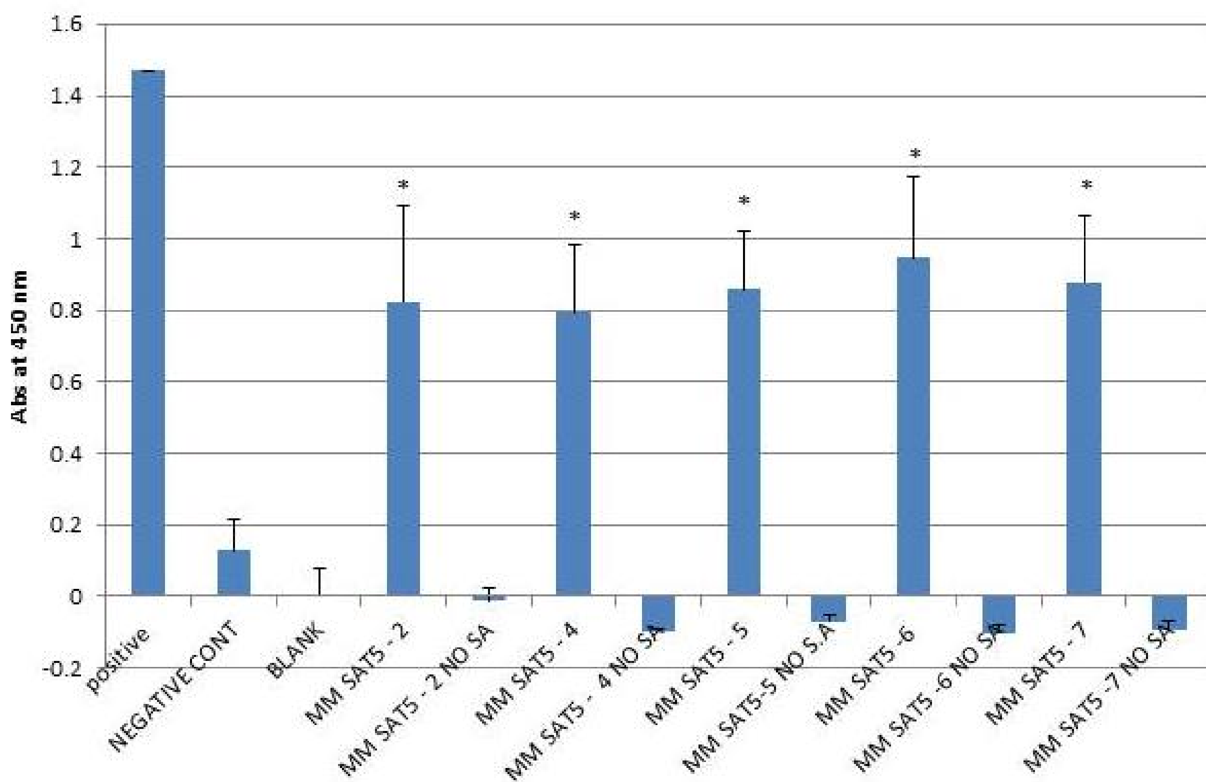

Figure 6. M13 ELISA test of clones 2,4,5,6 and 7 for binding to *S. aureus*, type 5. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation.

composition. Capsule types 5 and 8 are most commonly seen in hospital acquired infections (Nanra *et al* 2012).

We tested *S. aureus*, type 8 to determine if the phage peptides were specific to capsule type 5, or only to *S. aureus*. We also tested for binding to *S. epidermidis*, a non-pathogenic strain of staphylococcus and to *E. coli*, and a common intestinal Gram negative bacteria.

Figure 7 shows that, in addition to binding to *S. aureus*, type 5, MMSAT5-2, MMSAT5-4 and MMSAT5-5 are capable of binding to *S. aureus,* type 8 bacteria. There also appears to be some binding in the negative control wells. The ELISA done to test the phage clones for non-specific binding to *S. epidermidis* bacteria stain revealed that there was no binding to the non-pathogenic strain for most clones (Fig. 8). The five selected phage clones were also tested for binding to *E. coli* bacteria (Fig.9). In this case, there appears to be slight binding for all clones. However, this binding is much less than that seen for *S. aureus*.

IV) DISCUSSION

We hypothesized that PhD -7 phage library panned against *S. aureus* type 5 capsular polysaccharide will result in the selection of phage displaying the peptides bind specifically to *S. aureus* bacteria. The specificity of the phage displayed peptide binding to the bacteria was confirmed using phage ELISA. Our data showed that five out of the ten phage peptide clones selected from the panning round three titer plate bind specifically to *S. aureus* type 5 capsular polysaccharide. These clones include: MMSAT5-2, MMSAT5-4, MMSAT5-5, MMSAT5-6 and MMSAT5-7.

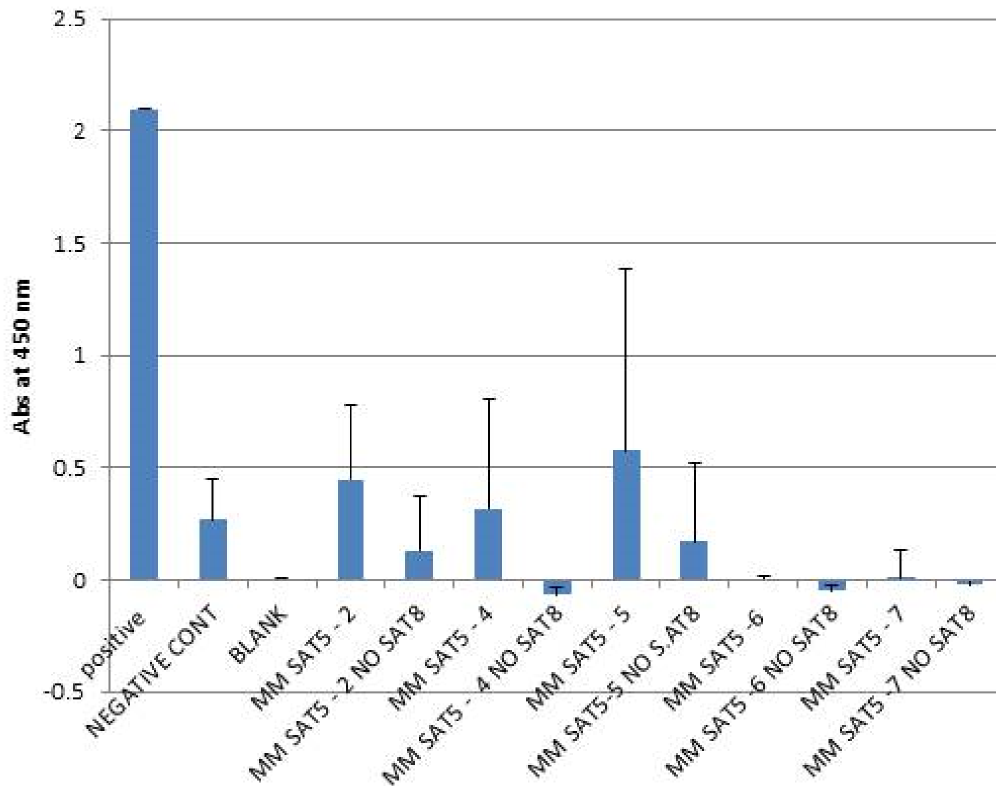

Figure 7. M13 ELISA test for binding to *S. aureus*, type 8. In this ELISA the clones were tested for non- specific binding to *S. aureus,* type 8 Bacteria. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation, and is one of three similar experiments (see Appendix 1).

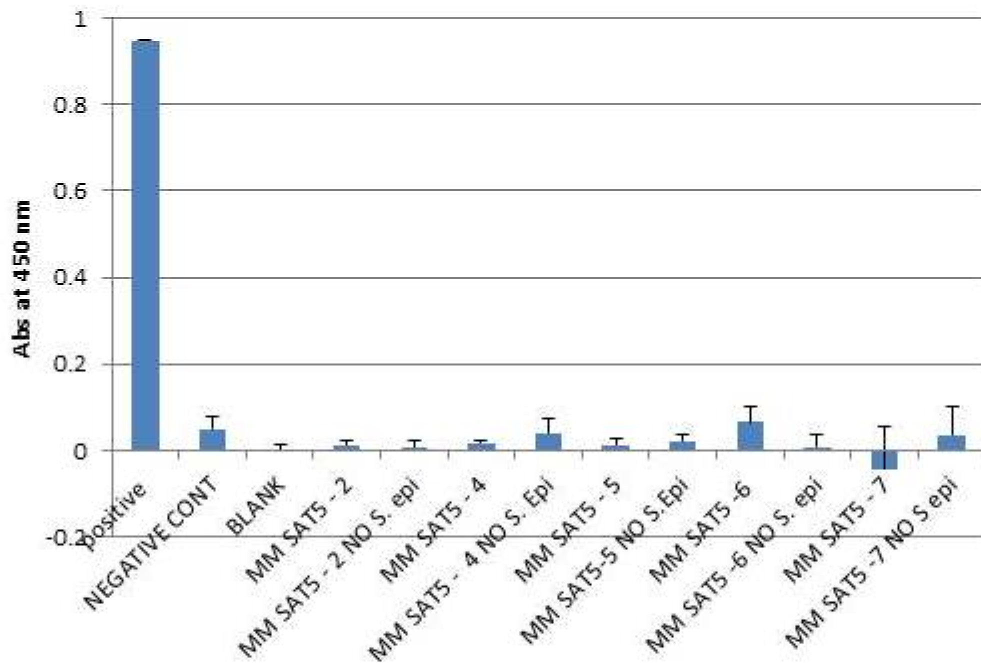

Figure 8. M13 ELISA test of clones 2,4,5,6 and 7 for binding to *Staphylococcus epidermidis*. In this ELISA the clones were tested for non-specific binding to *S. epidermidis* bacteria. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation, and is one of three similar experiments (see Appendix 1).

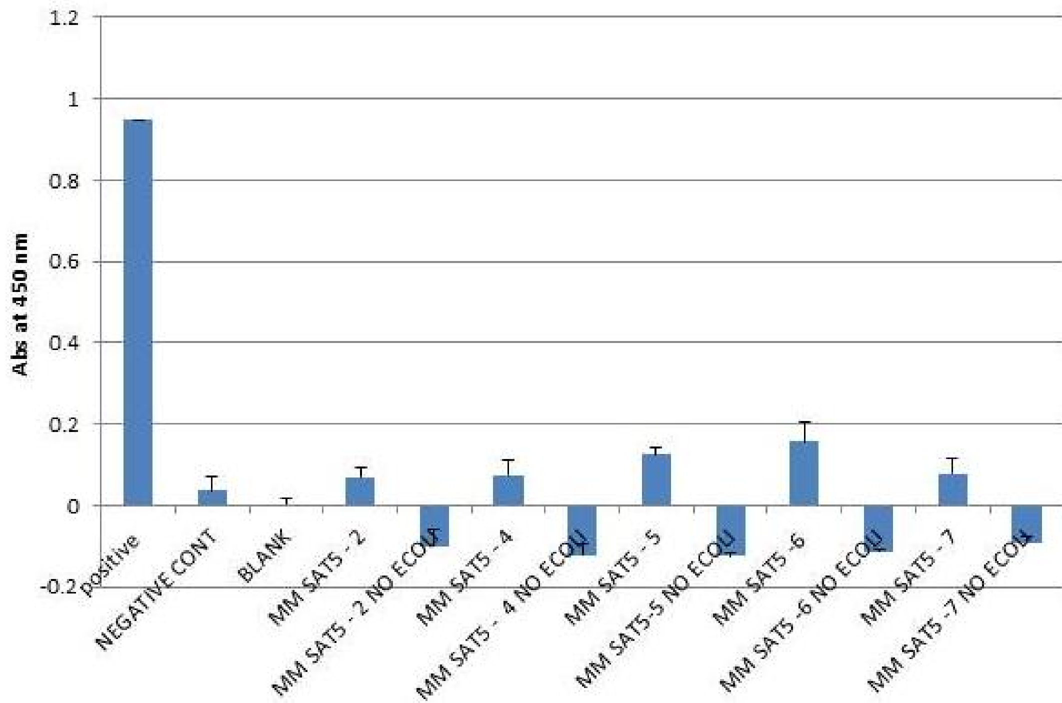

Figure 9. M13 ELISA test for clones 2,4,5,6 and 7 for binding to *Eschericia coli*. In this ELISA the clones were tested for non- specific binding to *E. coli* bacteria. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation, and is one of three similar experiments

S. aureus is an important human pathogen due to the wide spectrum of infections it causes and the rise in antibiotic resistance (Harris *et al*., 2002). Some of the infections are

superficial and mild; however, others are fatal with mortality rates as high as 25% within 30 days of infection, despite appropriate treatment (Lee *et al* 2004). Three to four percent of infants born with very low birth weight in the United States are diagnosed with S. *aureus* meningitis, bacteremia and neonatal sepsis resulting in a 26% mortality rate per year (Thammavongasa *et al*., 2014)

It is estimated that 20 to 50% of the human population are asymptomatic carriers of the bacteria in their nares. This is an important factor that contributes to the high rate at which the bacteria infection spreads. Whenever there are lapses in immunity or in the event of breach of the skin or the mucus membrane, the bacteria invade the body producing different infections, including osteomyelitis, septic arthritis, endocarditis, and bacteremia which is usually fatal. (Torre *et al*., 2015).

The Bacteria poses a great threat to public health globally since as there are now MRSA strains that are also resistant to vancomycin. These strains cause infections that have no cure. Because the resistance gene is carried on the plasmid, the rate of resistance spread is very rapid. (Daini and Akano, 2009). The mode of action for both beta-lactam ring containing drugs, for example methicillin, and the glycopeptides, including vancomycin, is to prevent bacteria cell wall formation. This causes bacteria death by autolysis due high osmotic pressure. MRSA has altered the target for these drugs by forming penicillin binding protein that have a very low affinity for the antibiotics, preventing binding and inhibition of cell wall formation. On the other hand, vancomycin

resistant strains prevent antibiotic action by producing more peptidoglycan and forming thicker cell wall that traps the drug.

MRSA have spread worldwide as a nosocomial pathogen (Harris *et al.,* 2002). They are especially endemic in many hospitals in America and Europe, representing about 30%- 35% of the total clinical isolates. Colonized patients are the main reservoir but the infections rapidly spread by poor hand sanitation (Braves *et a*l., 2015). The emergence and the spread of the strains with multiple antibiotic resistances necessitate more efforts to discover an alternative therapeutic compound. Despite more resources directed towards discovery of a novel S. *aureus* vaccine, the currently available vaccines are not effective at preventing disease. Many investigators think that the murine model in which most studies are carried out is not fully representative of human immune system. This view is supported by an observation that most of the vaccines that have appeared to work in the mouse animal model have failed at the clinical trial level (Reviewed in Fowler and Proctor, 2014).

Previously, our lab was involved in production of monoclonal antibodies directed against *S. aureus* capsular polysaccharide type 5 and 8 (Fagan et al 2004). These antibodies were confirmed to be type specific, and to promote opsonophagocytosis using human white blood cells (Royal and Fagan, 2008). However, due to the increasing evidence from many studies that murine antibodies are ineffective in humans, there is little hope the antibodies will stand the test of clinical efficacy.

In these studies we are investigating the possibility of selecting for peptides that bind specifically to *S. aureus*. In numerous studies peptides developed using phage display seem to be working with reasonable success in treating other diseases and

infections (Ribeiro *et al*., 2003). Phage display is a powerful affinity selection method for the discovery of novel peptides that bind with specificity to a target, such as receptors or other cell proteins. Phage display libraries are constructed by inserting a random DNA sequence upstream the gene coding for bacteriophage coat protein, leading to display of more than 10^9 random peptides fused to the coat protein (Nielsen *et al.,* 2016)

Phage display technology has previously been used to generate novel therapeutic peptides used to treat different disease and infections. For example, a novel decapeptide with antimicrobial activity against *Candida albicans* was identified using this method (Hong *et al.,* 1998). This peptide solved the problem of antifungal drug resistance and drug toxicity problems caused by fungicidal agents used to treat systemic mycosis. Zhou and colleagues also showed that phage display technology could be used to select tumor cell binding peptides (Zhou *et al.,* 2015). These peptides specifically bound to ovarian carcinoma cells both in vivo and in vitro and were confirmed able to inhibit cell viability, proliferation, migration, inversion and adhesion.

In this study, we proposed to use the PhD-7 phage library (New England BioLabs, Ipswich, MA) and the phage display cycle to select for phage displaying peptides that bind specifically to *S. aureus,* type 5, capsular polysaccharide. It is hoped that in future studies an antibacterial substance may be coupled to a specific peptide to generate an alternative therapeutic compound for treating the VRSA bacterial infections. Why not just use link the toxic peptide to the antibodies that were already generated?

Rao and coworkers used phage display to isolate peptides binding to *S. aureus* (Rao *et al*., 2013). In their studies the library was twice panned against other strains of bacteria, including *E. coli* and *S. epidermidis* to eliminate non-specifically binding

peptides before selecting for *S. aureus* specific phage. This strategy was referred to as subtractive panning. In this study the phage library peptide was first pre- adsorbed to casein blocking buffer to deplete the phage having peptides non-specifically binding to casein (Zhang *et al.,* 2002).

The *S. aureus* used as target in our protocol was grown in Columbia broth agar +2% NaCl at 37^0c incubating overnight. The cells were washed with PBS by centrifugation and formalized to kill the bacteria. Trypsinization of the cells served to destroy other surface membrane proteins, including protein A that may cause non-specific binding of the peptides (Thakker *et al*., 1998 and Sinha *et al.*, 1999). This also inhibited the selection of phage that bind to proteins on the surface of the bacterium. Panning was performed by incubating previously pre-adsorbed phage (to remove casein binding phage) with the formalized and trypsinized *S. aureus,* type 5 coated on a 96 well Poly –L- lysine plate. As the type 5 capsule is negatively charged, the poly-L-lysine coating used to bind to negatively charged cells in culture also serves to bind the bacterium to the plate. Blocking the bacteria coated wells with casein blocking buffer served to prevent the phage from binding non-specifically to the plate. After the phage was allowed to bind to the target, non-binding phage was discarded and the bound phage eluted with elution buffer. Three rounds of panning were done produce a more enriched target specific phage population (Table 1) (Thakker *et al.,* 1998 and Sinha *et al.,* 1999).

In studies by Heilmann and coworkers, phage was panned against purified immobilize platelets and the secondary phage stock was panned against human albumin serum (HSA) as a negative control to eliminate the phage non-specifically binding to HSA (Heilmann *et al*., 2002). Panning against HSA may have been a very important step,

as the selected peptide will be introduced into human body as a therapeutic compound. It would be desirable for the phage selected not to demonstrate non-specific binding to HSA and other blood components. In our study we initially blocked the wells with casein, but this was alternated with panning against wells blocked with BSA (bovine serum albumin). Previous work from this lab have shown that phage peptides selected for binding to BSA will also bind to HSA (Cortes *et al*., 2013). Adding BSA block to the second panning step, not only selected against any casein specific phage remaining after the first panning, but also eliminated phage that might bind to HSA.

Phage titering was done between each panning round and after amplification of the phage to ascertain if there was enrichment of the peptides being isolated (Heilmann *et al.,* 2002). The method used for titering the phage, was a plaque forming assay. *E. coli* bacteria strain ER2738 was grown in LB broth supplemented with tetracycline to select for F^+ cells as the F factor in this strain contains a mini-transposon for tetracycline resistance. The *E. coli* overnight culture was infected with eluted phage in different serial dilutions and plated with top agar and X-gal and IPTG on a pre-warmed LB Tet plate. The soft agar (top agar) served to restrain free movement of the phage produced, insuring that every clone is maintained at a fixed point. The IPTG induces *LacZ* gene contained in the M13 phage to produce a beta-galactosidase enzyme. This enzyme metabolizes X-gal, which is an analogue of galactose, into lactose and glucose which produces a bright blue color. Consequently, the library phage clones forming plaques on LB Tet plate are identified as blue plaques (Figure 1). The plaques are counted and multiplied by the dilution to obtain the phage titer in plaque forming units per ml (Barbas *et al.,* 2011).

Amplification of the phage was done to increase the population of the phage eluted every after panning step. It was also done on the plaques picked after panning round 3 to generate sufficient quantities of the phage needed for phage ELISAs and DNA sequencing. Amplification is the method of phage propagation that exploits the naturally existing *E. coli* to virus parasitic relationship. Ff filamentous phage is naturally capable of infecting and propagating in *E. coli* bacteria bearing the F-pillus. This process has been harnessed and used *in vitro* to propagate the bacteriophage, and at the same time, to increase the synthesis of peptides, antibodies, antibody fragments or proteins displayed on the phage surface. The DNA segment of interest coupled to the phage coat protein DNA is multiplied inside the bacteria and when phage is produced, the proteins are displayed on the phage surface for easier affinity selection. M13 phage replicate quickly, producing titers of up to 10^{13} pfu/ml or more (Warner *et., al* 2014). To amplify the phage, an overnight *E. coli* culture grown in LB broth with Tet was inoculated into Terrific Broth and inoculated with phage (Barbas *et al.,* 2011). After incubation, the bacteria were removed by centrifugation and the phage collected from the supernatant using PEG precipitation.

Warner and coworkers investigated the parameters that optimize phage production during the amplification step (Warner *et al.,* 2014). These studies revealed that increasing the agitation rate from 150 -240 rpm during incubation period increased the gas exchange, resulting in the production of high phage yields. Media composition was also cited as crucial factor, with Terrific Broth being preferred over LB broth because of it of its higher energy density. Temperature was another significant variable cited. In low temperatures of 29^0C and elevated temperatures of 30^0C phage production

increased by 1.5-fold as compared 32^{0}C. Our amplification process used the suggested protocol developed by these studies (Warner and coworkers). Amplification was performed in Terrific Broth with shaking at 210 rpm. However, due to the use of a shared incubator, the incubations were done at 37^{0}C. However, amplification did result in high phage titers (Table 1).

The culture in this study was incubated for 4-5 hours. Warner and colleagues argued that harvest time length is not a crucial factor (Warner *et al.,* 2014). However, as we had low titers following the first two amplifications, it is perhaps a good idea to more closely monitor this parameter in the future with the goal of increasing the phage titers. The phage was then separated from the bacteria cells by centrifugation, and precipitated by adding polyethylene glycol (PEG) and then resuspended in TBS (Warner *et al.,* 2014 and (Barbas *et al.,* 2011).

To identify phage clones with desired binding characteristics, ELISA screening assays were performed. After three rounds of *in vitro* panning, ten plaques were randomly chosen from the titration plate. These clones were labeled MMSAT5-1 through MMSAT5-10. These clones were first tested using phage ELISA to detect phage with binding specificity to *S. aureus,* type 5. In the phage ELISA, a 96 well poly-L- lysine microtiter plate was coated with ligands, as described for the panning step. M13KE phage was used as the negative control, as this is the phage used to construct the library. As M13KE does not express peptides bound to P3, any binding seen in the negative control wells would result from proteins found naturally in the phage, rather than from the displayed peptides. The blanks wells contained all reagents, except phage. The purpose of the blank was to test if the anti M13/HRP conjugate antibodies bind to the

target in the absence of phage. The NO SA wells were the wells without ligands and they served to test if the phage non–specifically binds to the casein blocking buffer or the plastic wells. After incubations to allow phage binding and a wash to remove non-specific phage, the plate was incubated with anti- M13/HRP conjugate antibodies. Horse radish peroxidase conjugated to the anti M13 antibodies is an enzyme that metabolizes the TMB substrate to produce a colored compound. A stop solution is added as soon as a stable color is formed and color intensity is read by absorbance using a spectrophotometer.

Our ELISA results showed that five clones appeared to bind specifically to S aureus, type 5 (Figures 2-6, A1 and A2). Although in the first round of ELISA clone 1 (MMSAT5-1) also showed very impressive binding to *S. aureus* bacteria (Figure 2 and 3), in subsequent ELISAs (data not shown) it appeared to bind non-specifically to other bacteria strains including *S. aureus,* type five and *S. epidermidis*. No further analysis for this clone was necessary therefore. The five clones that were further tested were clones MMSAT5-2, MMSAT5-4, MMSAT5-6 and MMSAT5-7. These clones were selected and tested for non-specific binding to *S. aureus* type 8, *S. epidermidis*, and *E. coli*. In tests examining the reactivity of these clones with *S. aureus*, type 8 (Figures 7, A8 and A9), *S. epidermidis* (Figures 8, A5, and A6), and *E. coli* (Figures 9, A7 and A8), none of the clones had significantly different absorbance results when incubated with the bacteria indicated, than was seen in the blank wells. This data demonstrates the phage selected bind specifically to *S. aureus*, type 5, and not to the other organisms tested.

In future studies the genes for the cloned peptides will be inserted into vectors bound to a toxin, in the hope that the peptide/toxin conjugate may be used to treat

Staphylococcus infections. There are many potential toxins that may be useful in this capacity. In a study by Hong and colleagues (Hong *et al.,* 1998), a positively charged peptide was isolated due to its toxicity to *C. albicans*. The peptide was also shown to be toxic to *S aureus*. It was argued that these peptides bound to and interacted with fungi and bacteria because the cell membrane in both cases is negatively charged due to the presence of phospholipids. Because of phosphatidyl-glycerol and cardiolipin in bacteria cell membranes, the cells are said to be more negative than the fungal cells. As a result, the positively charged peptides are thought to interact with bacteria membranes enhancing the permeability of the lipid membrane. Fortunately, the peptides did not demonstrate any red blood cell hemolysis. The small size, structural properties and hydrophobicity of the peptide are among the factors thought to have contributed to the peptides' low level of hemolytic activity when used in the body.

Another study looking for antibacterial peptides conducted by Ribeiro and coworkers, showed a peptide, "RIP *YSPXTNF*" derived from *Staphylococcus xylosus* and its synthetic derivatives "*YSPWTNF*" and "*YSPITNF*" having antimicrobial activity. The peptide is thought to function in vivo by preventing *S. aureus* and *S. epidermidis* bacteria from producing toxins and biofilm. This makes the bacteria less pathogenic and more susceptible to human immune defense system response. (Ribeiro *et al*., 2003)

Human beta- defensin antimicrobial peptide may also be a good example of the molecule to be couple to the peptides selected in our study. Available data shows that N-terminus of human defensin -3 joined to mannose binding lectin with a linker GGG showed very good Bactericidal activity when tested against MRSA in murine model.

Antimicrobial peptides are immune effector molecules formed as a component of the innate immune defense system (Li *et al*,.2010).

Another molecule that has shown potential therapeutic efficacy in treating MRSA is staphylolysin. This is a staphylolytic endopeptidase secreted by *Pseudomonas aeruginosa*. In a study where this molecule was applied as a topical treatment of an MRSA infection causing keratitis, the infection was totally cleared and no histological side effects were observed. (Barequet *et al*., 2012)

Conclusion and Future Directions

Phage clones specific for *S. aureus*, type 5, were selected from the Ph.D.-7 library (New England BioLabs, Ipswich, MA). Five specific clones (SAT5 -2, SAT5-4, SAT5-6 and SAT5-7) were also tested against S aureus, type 8, S. epidermidis, and E. coli and shown to not bind to these bacteria. The clones SAT5 -2, SAT5-4, SAT5-6 and SAT5-7 will, in future studies, be tested against *S. aureus*, type 5, capsular carbohydrate by an ELISA assay. The clones selected at that later point will be sequenced and the DNA sequence translated to amino acid sequence. The amino acid sequence will be used to produce pure synthetic peptides. Peptide ELISAs will be done to confirm binding specificity before the peptide is coupled to antibacterial molecules. It is hoped that the compound generated in this way will serve as an alternative therapeutic drug to mitigate the current problem experienced due to antibiotic resistance of the *S. aureus* infections.

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VI) APPENDIX

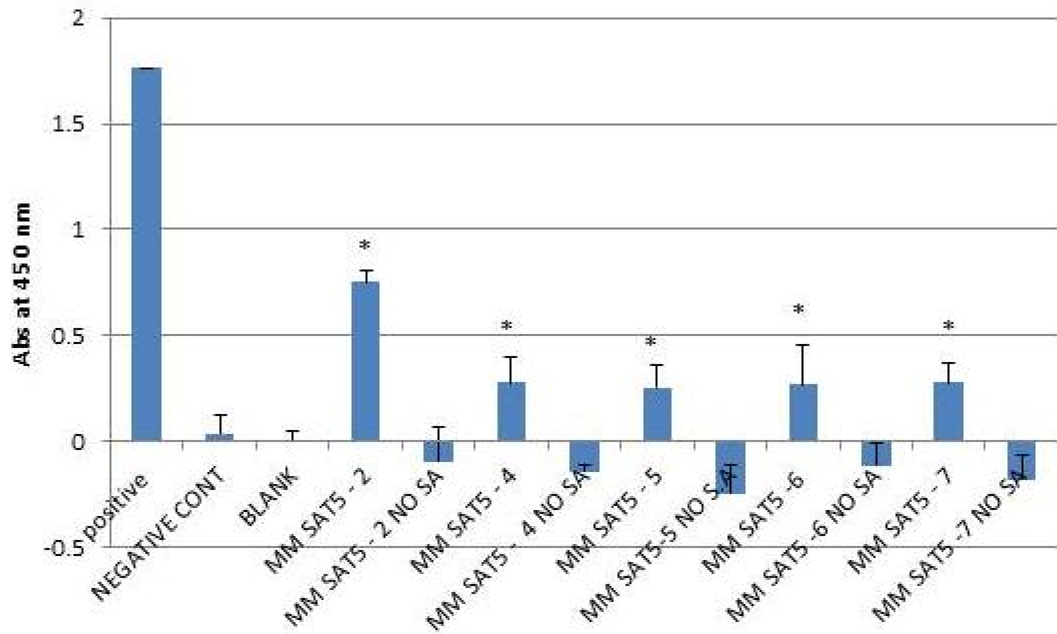

Figure A1. ELISA testing binding of clones 2, 4, 5, 6, and 7 for binding to *S. aureus*, type 5.

The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation

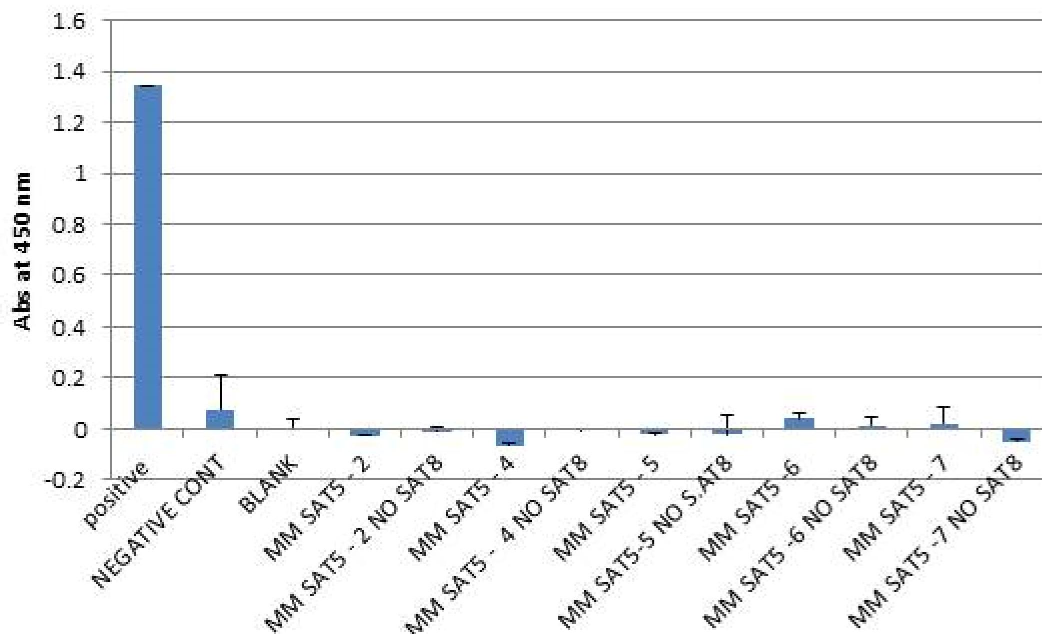

Figure A2. ELISA testing binding of clones 2, 4, 5, 6, and 7 for binding to *S. aureus*, type 5.

The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation.

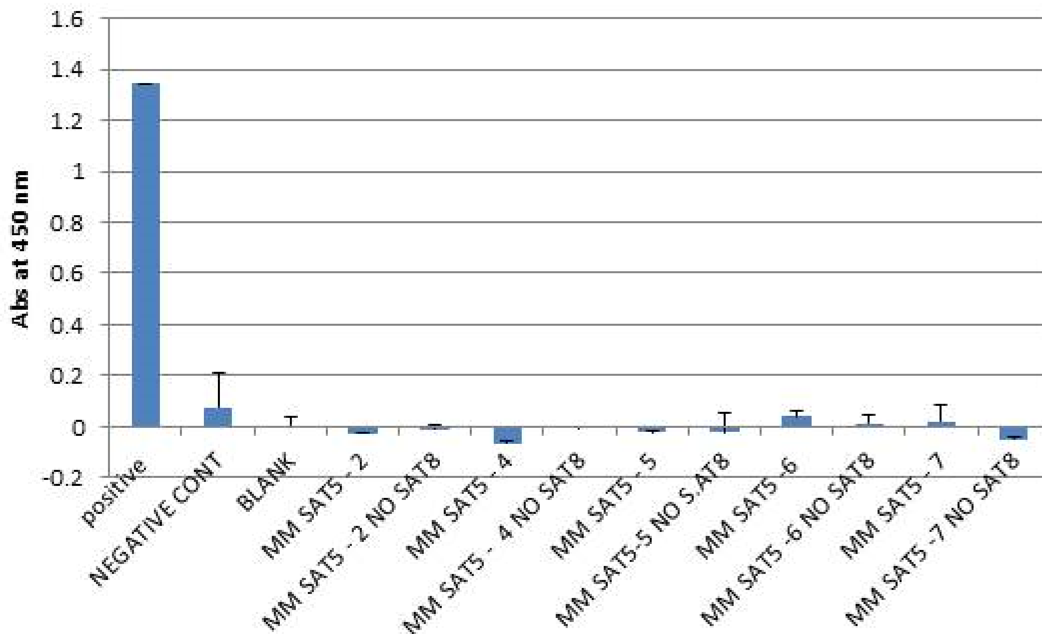

Figure A3. ELISA testing binding of clones 2, 4, 5, 6, and 7 for binding to *S. aureus*, type 8. In this ELISA the clones were tested for non- specific binding to *S. aureus,* type 8 Bacteria. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents

data performed in triplicate (with the exception of the positive control) +/- Standard Deviation, and is one of three similar experiments.

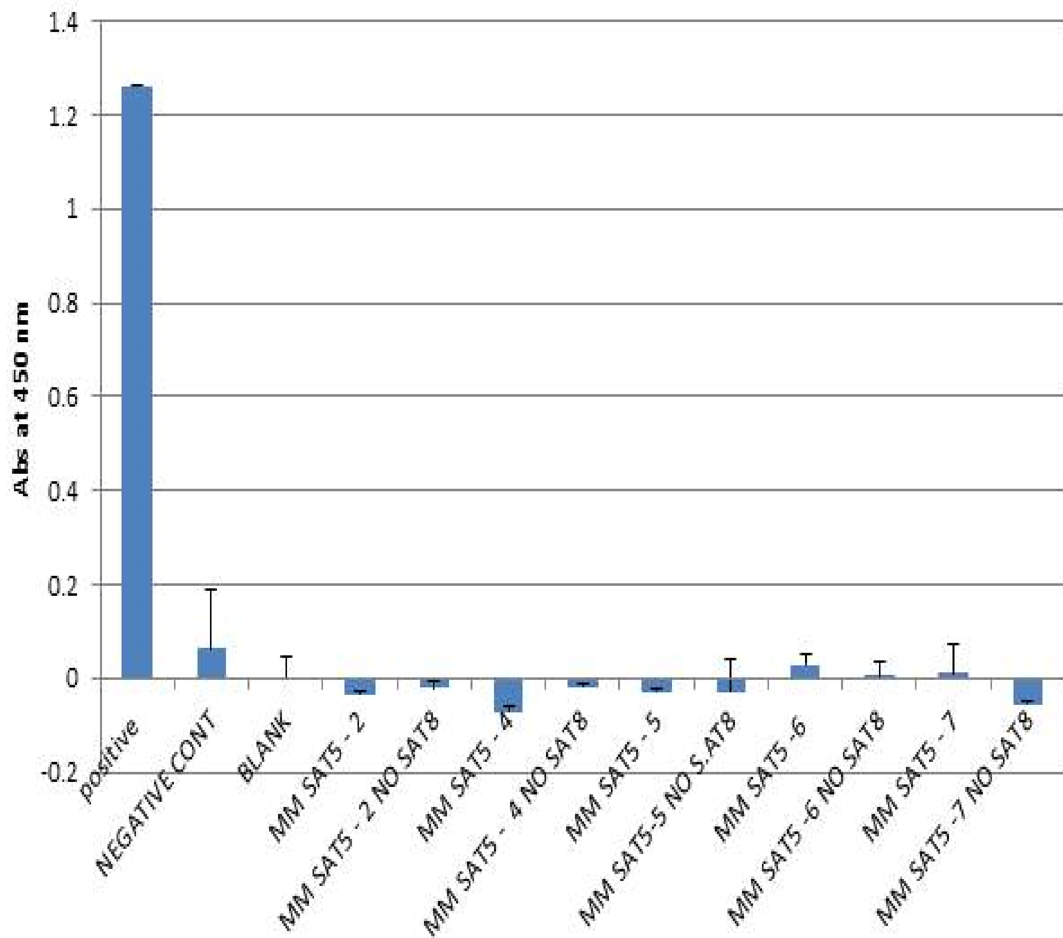

Figure A4. ELISA testing binding of clones 2, 4, 5, 6, and 7 for binding to *S. aureus*, type 8. In this ELISA the clones were tested for non- specific binding to *S. aureus,* type 8 Bacteria. The ELISA was performed as described in Figure 2. AmpR (previously

amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation, and is one of three similar experiments.

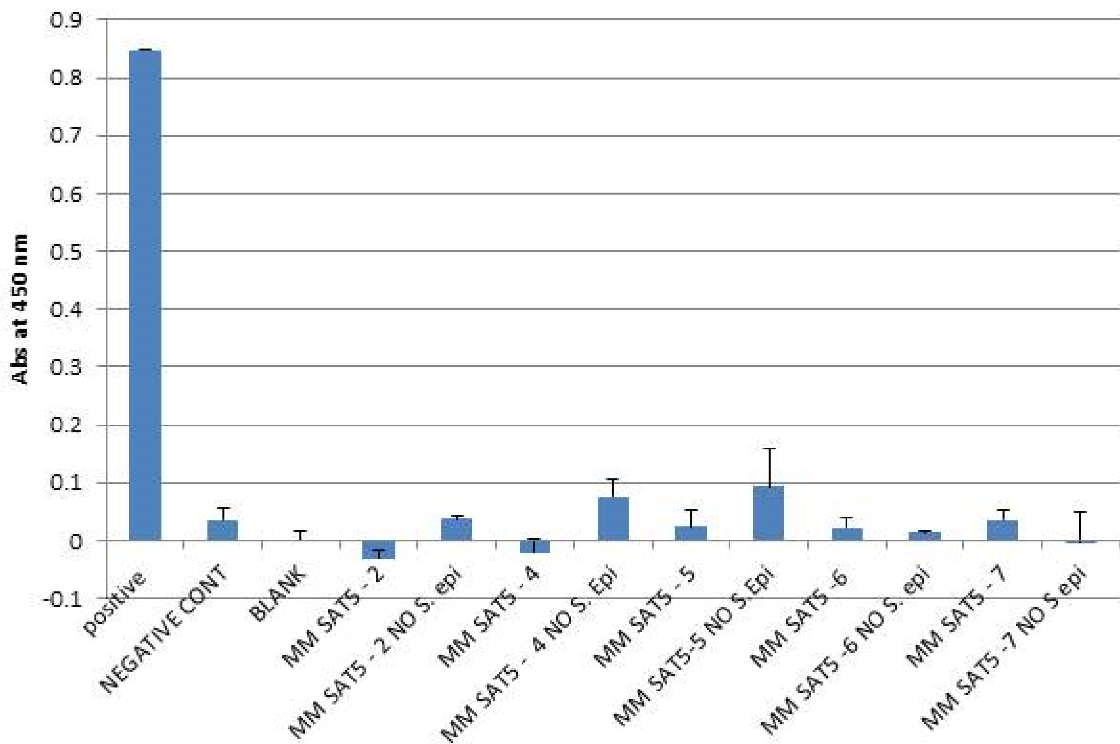

Figure A5. ELISA testing binding of clones 2, 4, 5, 6, and 7 for binding to *S. epidermidis*. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation, and is one of three similar experiments.

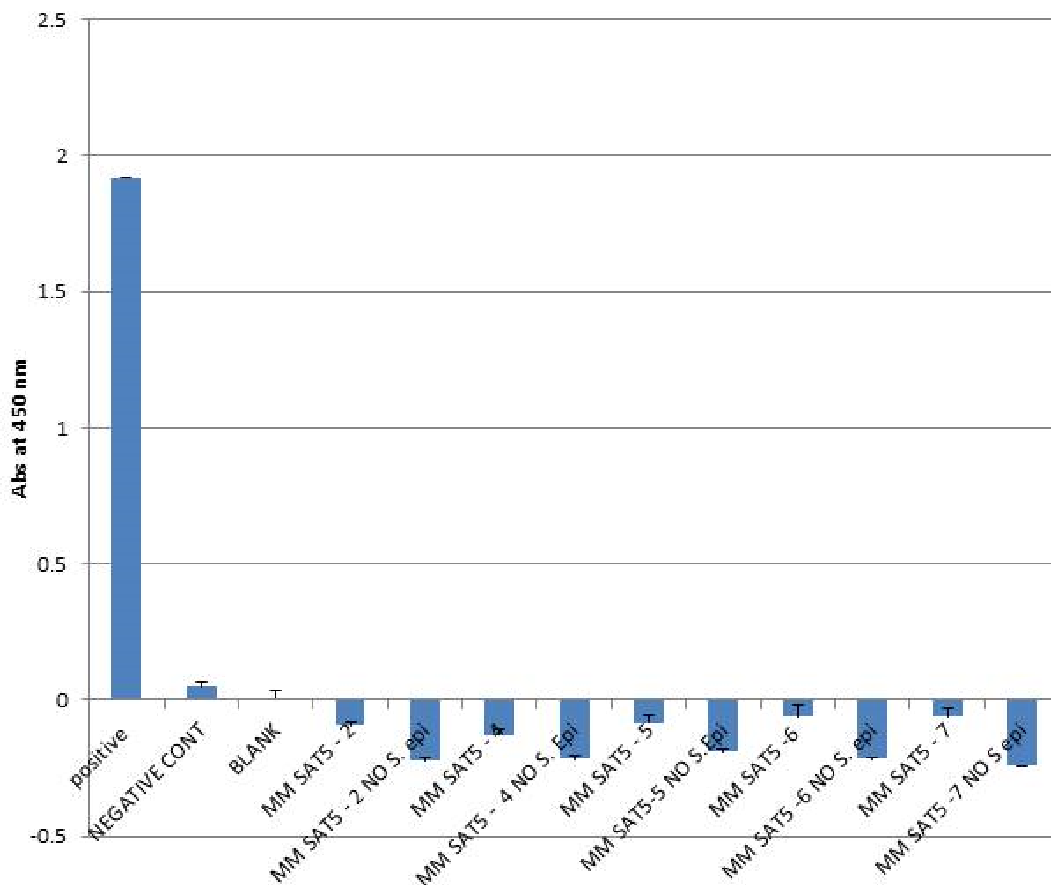

Figure A6. ELISA testing binding of clones 2, 4, 5, 6, and 7 for binding to *S. epidermidis*. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation, and is one of three similar experiments.

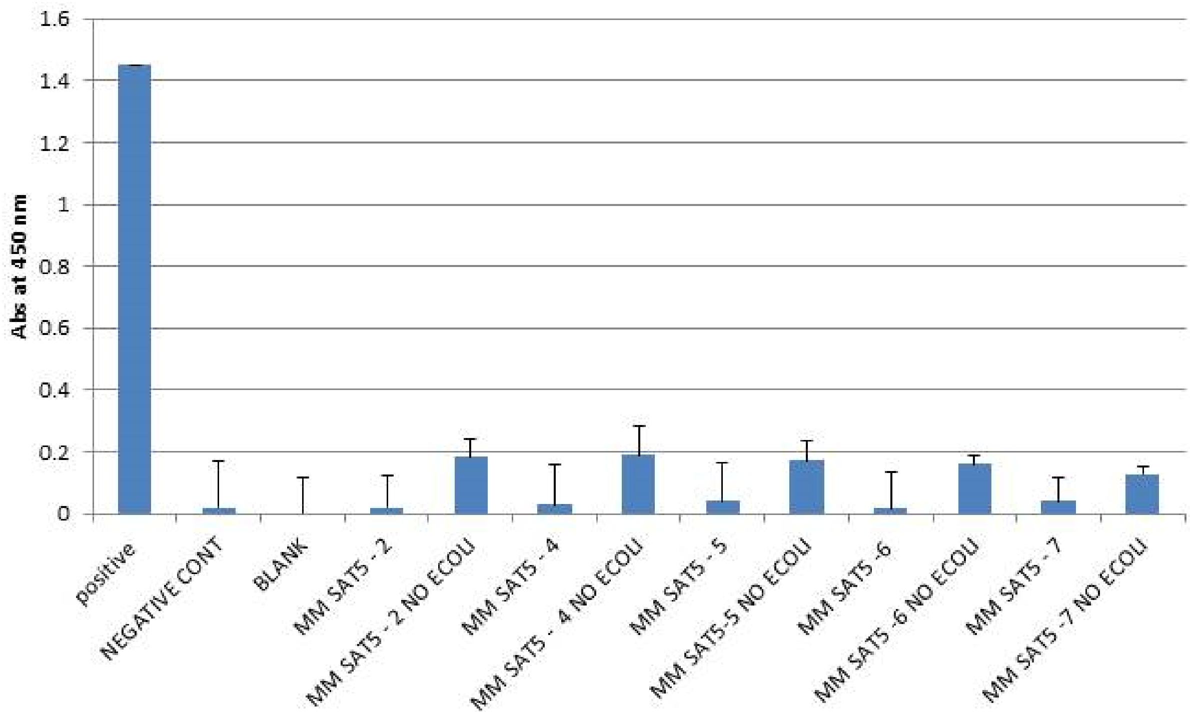

Figure A7. ELISA testing binding of clones 2, 4, 5, 6, and 7 for binding to *E. coli.* .In this ELISA the clones were tested for non- specific binding to *E. coli* bacteria. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation, and is one of three similar experiments

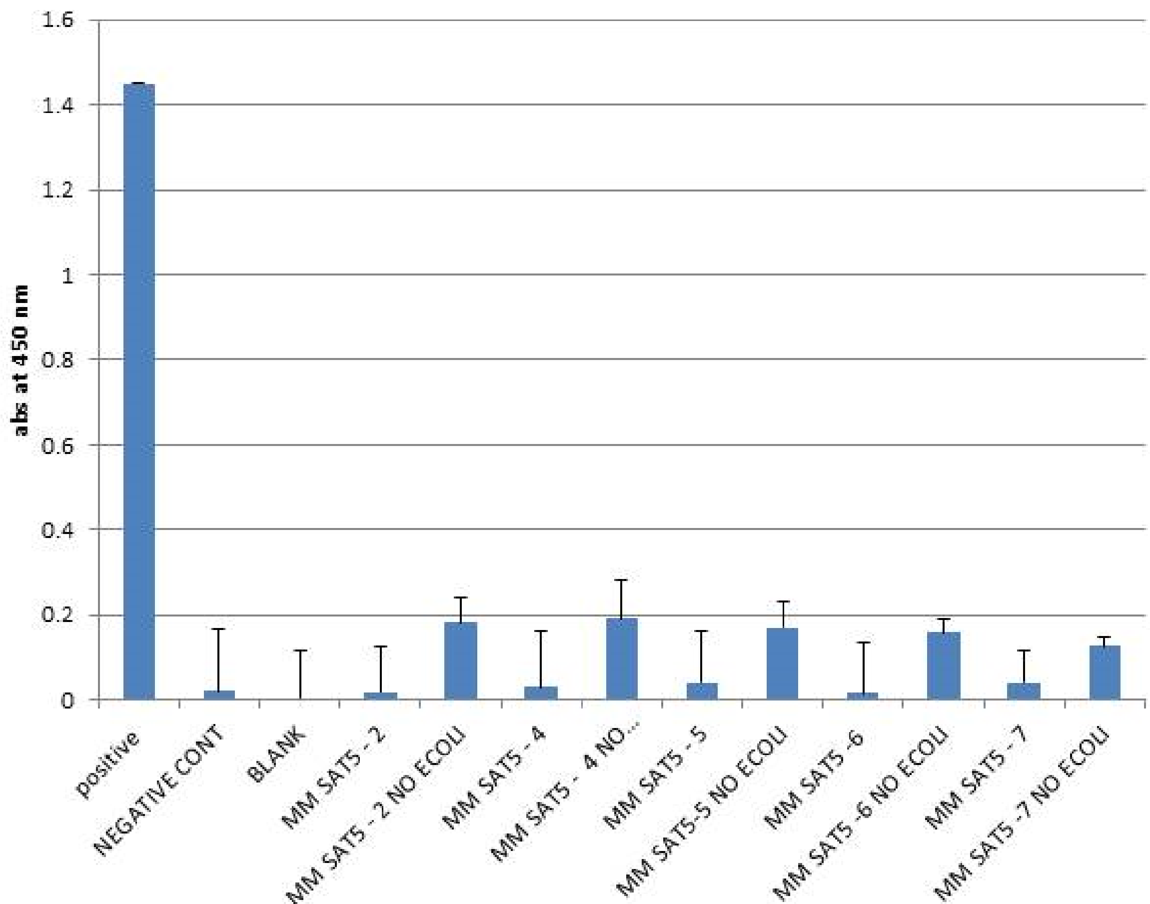

Figure A8. ELISA testing binding of clones 2, 4, 5, 6, and 7 for binding to *E. coli.* In this ELISA the clones were tested for non- specific binding to *E. coli* bacteria. The ELISA was performed as described in Figure 2. AmpR (previously amplified phage from the PhD-7 library) was diluted in $NaHCO_3$ for the positive control well. The negative control was M13KE (phage with no peptide). The blank (all reagents, except the phage) was subtracted from all other conditions. This experiment represents data performed in triplicate (with the exception of the positive control) +/- Standard Deviation, and is one of three similar experiments