Testing glycomimetic compounds for their ability to disrupt capsular polysaccharide production in type 5 *Staphylococcus aureus*.

by

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Thesis Abstract

The potentially fatal nature of *Staphylococcus aureus* is exponentially enhanced by its incredible ability to evade medical treatment. At the current time *S. aureus* has developed resistance mechanisms to every class of antibiotics that are supposed to inhibit or kill the bacterium. Physiologically *S. aureus* is able to evade host immunological defense mechanisms due to the presence of a structure known as the *capsular polysaccharide*. Inhibiting production of the capsular polysaccharide provides a novel approach to combating antibiotic resistant *S. aureus*. By targeting the synthesis of the sugars that make up the capsule, compounds can be developed that may inhibit capsule formation. These sugars are specifically targeted *via* glycomimetics. The mimetic compounds are tested to determine their ability to inhibit production of the capsular polysaccharide of *S. aureus.* The ability of the mimetic to block capsular polysaccharide production can be measured by analyzing the binding of monoclonal antibodies to the capsule.

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Table of Contents

Title Page……………………………………………………………………………......i

Signature Page…………………………………………………………………………ii

Abstract……………………………………………………………………………...iii

Acknowledgments…………………………………………………………………..iv

Table of Contents……………………………………………………………………v

List of Figures…………………………………………………………………….....vi

Introduction…………………………………………………………………………..1

Statement of Problem………………………………………………………………21

Results………………………………………………..…………………………..22

Discussion…………………………………………………………………………..25

Conclusion…………………………………………………………………………27

Experimental………………………………………………………………………..28

List of Figures

Figure 1……………………………………………………………………………..17

Figure 2……………………………………………………………………………..18

Figure 3……………………………………………………………………………..22

Figure 4……………………………………………………………………………..41

Figure 5……………………………………………………………………………..42

Figure 6……………………………………………………………………………..24

Figure 7……………………………………………………………………………..43

Figure 8……………………………………………………………………………..44

Figure 9……………………………………………………………………………..29

Figure 10……………………………………………………………………………45

Introduction:

Diseases caused by *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that can cause a diverse array of diseases. For instance, *S. aureus* is most frequently the cause of skin and soft tissue infections. The complications due to such infections range from minor eruptions through infected ulcers and cellulitis to severe impetigo. *S. aureus* is also a frequent invader of surgical and other wounds, sometimes leading to sepsis.[1] In wound infections *S. aureus* has an invasive potential to induce osteomyelitis, endocarditis, and bacteremia, leading to secondary infections in any of the organ systems. Staphylococcal infections occur most frequently when the skin or mucosal barriers are breached, following insertion of a foreign body and in hosts with compromised immune systems.[2] *S. aureus* is one of the more common causes of prosthetic valve endocarditis and an occasional agent of post-neurosurgical meningitis.[1] Additionally, *S. aureus* produces numerous exotoxins, some of which cause diseases such as toxic shock syndrome and food poisoning.[3]

Origins of Treating *S. aureus* Infections

In the 1930's medicinal treatment did not include antibiotic therapy, as penicillin had not yet been discovered. However, maggot therapy was introduced into American medicine as a means to clean wounds. Maggots only feed on infected tissue, and cause no harm to healthy tissue. Therefore, maggots would effectively clean wounds and save people from the consequences of serious infections. Shortly after the discovery of penicillin, maggot therapy was abandoned,[4, 5] as the antibiotic was regarded as the ultimate cure-all. Unfortunately, there are grave consequences for relying solely on

antibiotic therapy. From a medical standpoint, the main reason *S. aureus* is such a harmful pathogen is because of its incredible ability to evolve resistance mechanisms toward antibiotics. For instance, when penicillin was introduced in 1944 over 94% of *S. aureus* isolates were susceptible; by 1950 half were resistant.[1] The prevalence of penicillin resistant strains in hospitals began to rise as penicillin became readily available after World War II. Within a few years, most hospital isolates were resistant to penicillin.

Resistance to penicillin is due to the plasmid-mediated enzyme *penicillinase*, which is a β-lactamase that hydrolyzes the β-lactam ring of penicillin.[6] Today, nearly all *S. aureus* isolates in the United States are resistant to the various forms of penicillin.[7]

The Development of Methicillin Resistant *S. aureus* (MRSA).

The next major breakthrough in the treatment of antibiotic resistant *S. aureus* was the development of the drug class known as cephalosporins, which are very closely related to penicillins. These antibiotics were useful against *S. aureus* because of their stability in the presence of staphylococcal penicillinase. However, the most important antibiotic drug discovery of the 1960's dealt with how to replace the 6' phenylacetyl group of benzylpenicillin with other acyl substituents. This discovery provided a synthetic route for the synthesis of methicillin, nafcillin and the oxacillins. These compounds all have bulky 6' acyl groups that sterically hinder attack on the β-lactam ring, voiding the effect of penicillinase.[1] Unfortunately, the innate ability of *S. aureus* to adapt resulted in the first reported case of methicillin-resistant *Staphylococcus aureus* (MRSA) in 1961.[8, 9] Today the acronym MRSA is still used, but the drug oxacillin is used when testing *S. aureus* isolates for resistance to all β-lactam antibiotics.[10]

The usual mode of methicillin resistance is due to the acquisition of a specific staphylococcal chromosomal cassette. There are five possible chromosomal cassettes that code for methicillin resistance, which are referred to as staphylococcal cassette chromosome *mec* I-V (SCC*mec* I-V).[11] This mobile genetic element contains the *mec*A gene which codes for penicillin-binding protein (PBP2´), which confers an intrinsic resistance to all β-lactams and their derivatives.[8] Thus, the entire spectrum of cephalosporins is eliminated as a treatment option.[12] Methicillin resistance may also result from the development of hyper-β-lactamase-producing strains or the modification of existing penicillin-binding proteins.[9] In the laboratory setting it has been shown that methicillin resistance levels strongly depend on external factors such as temperature, osmolarity, the availability of divalent cations, and the composition of the growth medium. For example, incorporating NaCl into the growth medium and lowering the temperature increases the expression of MRSA strains.[8]

The established risk factors for MRSA infection include the following: recent hospitalization or surgery,[13, 14] prolonged hospitalization,[13, 14] care in an intensive health care unit,[14] residence in a long term healthcare facility,[13] dialysis,[13] internal medical devices,[13] prolonged antibiotic therapy, [14] and close proximity to a patient who is infected or colonized with MRSA.[14] Clearly, the risk factors indicate that MRSA was thought to be isolated to health care settings. Therefore, it was believed that *S. aureus* infections not associated with risk factors were still susceptible to methicillin. However, in 1993 the first report of a highly virulent MRSA strain in an Australian community presented in a patient lacking established risk factors.[15] It was not until 1999 when health care professionals in the United States began to notice the changing epidemiology of MRSA.

Four healthy children, in the Minnesota and North Dakota areas, became severely ill due to complications with *S. aureus*. These children had none of the established risk factors pointing to MRSA. Thus, it was assumed the infection was caused by methicillin sensitive *S. aureus*.[12, 16] Unfortunately, the result was treatment with an ineffective antibiotic (cephalosporin) and the deaths of the four children.[12] These fatalities set off alarm bells in the medical community, proving that MRSA had evolved and was no longer restricted to a hospital type setting. Ironically, if any of these children had been allergic to penicillin, their treatment options would not have included any β-lactam antibiotics. Furthermore, because of the very different treatment protocols for individuals with penicillin allergies, it is impossible to say if this outbreak of MRSA was limited to four cases.

The Categorization of MRSA

As more cases of MRSA were documented among healthy individuals without the established risk factors for MRSA acquisition, medical professionals were forced to reevaluate MRSA treatment. Currently, MRSA infections are now classified as either hospital-acquired (nosocomial) MRSA (HA-MRSA) or as community-acquired MRSA (CA-MRSA). These two strains of MRSA are genetically distinct from one another, resulting in different treatment procedures.[15] CA-MRSA isolates are generally susceptible to antimicrobials other than β-lactam antibiotics, and are more likely to retain susceptibility to multiple antibiotics.[12] HA-MRSA is typically resistant to a variety of non-β-lactam antibiotics.[7] Specifically, methicillin resistance arising from SCC*mec* I-III are commonly associated with HA-MRSA, whereas resistance coded by SCC*mec* IV and V tend to be associated with CA-MRSA.[11] It is evident that the evolving epidemiology of

MRSA can be compared to penicillin. Initially, penicillin resistant strains were associated solely with nosocomial infections, but resistance eventually spread into the community.[13]

The defining terms, hospital-acquired and community-acquired, do not necessarily mean the strains cannot cross their respective habitats. There are documented cases of CA-MRSA strains producing hospital-acquired infections.[15] Typically, the risk factors for becoming infected with a strain of CA-MRSA include the following: contact sports, shared bathing facilities, saunas and imprisonment.[17] Risk factors only highlight the most likely modes of transmission. Bacteria have an incredible ability to adapt and evolve and these processes are accentuated by the rapid rate of human movement within society. Therefore, it is easy to see how CA-MRSA can enter the hospital setting and consequently cause infection.

The Development of Vancomycin Resistant *S. aureus* (VRSA)

The development of methicillin resistance, in particular, has been a cause for concern among physicians and microbiologists in recent years, as effective treatment options are diminishing. Glycopeptides, such as vancomycin and teicoplanin, are often the therapeutic drugs of choice for serious MRSA infections. The latter is not approved for use in the United States,[18] leaving vancomycin as the only glycopeptide available.

The failures of vancomycin therapy have been highlighted by the emergence of *S. aureus* strains that are significantly less susceptible to vancomycin, resulting in vancomycin resistant *S. aureus* (VRSA).[19] Vancomycin was first produced in 1958, and maintained its status as an effective therapy for almost 40 years.[20] According to the Center for Disease Control and Prevention the situation changed in 1996, when the first

strain of *S. aureus* with reduced susceptibility to vancomycin and teicoplanin was isolated in Japan.[21] As of the end of 2006, there had been seven cases of VRSA in the United States: five cases from Michigan, and one each from Pennsylvania and New York.[22] In addition to Japan and the United States, VRSA has also been reported in France, South Africa, Brazil, and Scotland.[23]

To understand vancomycin resistance, one must understand how vancomycin works. Glycopeptides exert their antimicrobial effect by inhibiting synthesis of the *S. aureus* cell wall. Specifically, glycopeptides bind to D-alanyl-D-alanine residues of the murein monomer.[23, 24] This target is not very specific, because murein is a structural component of several *S. aureus* structures. This target is actually present in the completed peptidoglycan layers, the newly synthesized peptidoglycan chain, and on the cytoplasmic membrane. Glycopeptides are only effective when they bind to murein monomers in the cytoplasmic membrane. However, in order for glycopeptides to reach the binding sites on the cytoplasmic membrane, the drug must travel through about 20 layers of peptidoglycan. Thus, glycopeptides are unlikely to reach the targets on the cytoplasmic membrane because it ends up binding to the D-alanyl-D-alanine residues in the peptidoglycan layer. When transmission electron microscopy was used to examine the first clinical VRSA strain, there were approximately 35 layers of peptidoglycan around the cytoplasmic membrane. Thus, vancomycin is easily trapped in the peptidoglycan layers, voiding its therapeutic effect. The non-specific target of vancomycin severely compromises its effectiveness, even in non-VRSA strains.[23]

Additionally, PBP2´ is overproduced in teicoplanin resistant *S. aureus* as well as VRSA. However, PBP2´ overproduction tends to favor teicoplanin resistance. Some

teicoplanin resistant strains are treatable with vancomycin, and some strains of VRSA are treatable with teicoplanin. Thus, it is possible that the two glycopeptides may have different modes of action even though they target the same molecule. It is thought that teicoplanin inhibits transpeptidation, whereas vancomycin inhibits transglycosylation.[23] Analysis of two clinical examples from 2002 illustrates that vancomycin and teicoplanin resistance may or may not occur simultaneously. In June 2002, VRSA was isolated from a 40-year-old diabetic patient in Michigan. This strain of VRSA was highly resistant to vancomycin and teicoplanin.[21, 25] However, in September 2002, VRSA was isolated from a 70-year-old male patient, in Pennsylvania. This strain was not resistant to teicoplanin.[25] Thus, these cases support the claim that the glycopeptides vancomycin and teicoplanin may execute their therapeutic effects in different ways.

Other studies have suggested that structural and/or metabolic changes in cell wall teichoic acids may play a pivotal role in the resistance mechanism of glycopeptides.[24] Teichoic acids are charged polymers comprised of alternating negatively charged phosphate and positively charged alditol groups. Through modulation in their charge teichoic acids control autolytic activites and magnesium ion concentration within the cell wall.[9] By altering autolytic activities, teichoic acids can reduce the rate of cell wall degradation. The result is an excessively thickened cell wall, which consequently leads to a decreased susceptibility to glycopeptides.[24]

Other Antibiotics

Vancomycin and methicillin resistance is not required to occur in a sequential manner. There are VRSA isolates that remain susceptible to antibiotics which are ineffective for some MRSA isolates. There really is not a definite pattern to antibiotic

resistance. There are general trends, such as β-lactam resistance by MRSA is also seen in VRSA, since VRSA is simply a MRSA isolate resistant to vancomycin. The other classes of antibiotics present a game of hit or miss in terms of being an effective treatment option for MRSA or VRSA. Thus, diagnostic testing is conducted to determine antibiotic susceptibilities. As a general rule of thumb, VRSA tends to be resistant to the following classes of antibiotics: macrolides, aminoglycosides, tetracyclines, and fluoroquinolones.[17]

As explained earlier, methicillin resistance is due to the *mec*A gene. The gene *mec*A resides on SCC*mec*. SCC*mec* II and SCC*mec* III are of particular interest because these cassettes contain elements which encode resistance to additional antibiotics. SCC*mec* type II[26] also contains transposon 554 (Tn554) which encodes resistance against macrolides, clindamycin, and streptogramin B.[26, 27] SCC*mec* types II and III contains elements coding for resistance to aminoglycosides such as tobramycin and kanamycin. SCC*mec* type III also encodes for tetracycline resistance via the *tet*K gene.[26] Tetracycline resistance in *S. aureus* can occur in two ways. First, *S. aureus* develops a ribosomal protection mechanism to keep tetracycline from its target: the 30S ribosomal subunit. Secondly, *S. aureus* can develop an efflux mechanism to expel the antibiotic. These two resistance mechanisms are most often caused by the tetracycline resistance genes *tet*K and *tet*M.[28] Oddly enough, the 2002 VRSA isolate from Michigan was susceptible to tetracycline, and its derivative minocycline.[21]

Fluoroquinolones were initially prescribed for the treatment of Gram-negative bacterial infections. However, they are also used to treat Gram-positive bacterial infections caused by pneumococci and staphylococci. Unfortunately, *S. aureus* quickly

became resistant to flouroquinilones, especially in MRSA strains.[29] Even ten years ago 90% of MRSA isolates were resistant to fluoroquinilones.[30] A proposed reason for the fast pace of fluorquinilone resistance deals with colonization of *S. aureus* in humans. Approximately 60% of the healthy human population is permanently or intermittently colonized with *S. aureus*.[31] Colonized individuals many not show any disease symptoms due to *S. aureus*.[14] Therefore when colonized patients are treated for other bacterial infections with broad spectrum antibiotics such as fluoroquinolones, they are likely exposed to levels of antibiotics which are not lethal toward *S. aureus*. Therefore *S. aureus* is provided with an environment conducive for the development of antibiotic resistance.

Fluoroquinolone resistance develops as a result of chromosomal mutations in topoisomerase IV, DNA gyrase, or by induction of a multidrug efflux pump.[29] An alarming consequence to this pump is that it has the ability to pump out quaternary ammonium compounds, which are known for their disinfectant properties.[32] Ultimately, a class of drugs, embraced by the medical community for their quick action and success rate, became worthless for the treatment of *S. aureus*.[29]

Antibiotics Used for the Treatment of VRSA

Despite resistance to multiple antibiotics, VRSA is usually susceptible to a few other traditional antibiotic agents. For example, co-trimoxazole is a combination antibiotic containing trimethoprim and sulfamethoxazole. Usually, the glycopeptides are considered superior to standard dosages of co-trimoxazole. However, high dosages of co-trimoxazole have been helpful in combating drug resistant *S. aureus* infections caused by internal medical devices, especially orthopedic implants. This is especially helpful for two reasons: co-trimoxazole is available in an oral formulation, and can be used in long-

term therapy.[30] Most importantly, co-trimoxazole allows for patients to be treated outside of the hospital, unlike the glycopeptides, thus reducing medical cost.

Quinupristin/dalfopristin shows activity comparable to vancomycin against MRSA and can be used in the treatment of VRSA. However, its biocidal activity is void in macrolide-lincosamide-streptogramin-resistant strains.[17] Additionally, the medication causes severe irritation when administered intravenously. Thus, the only remaining way to administer the drug is *via* a central venous catheter. The complications with the drug and the very aggressive mode of therapy have lead to its abandonment. [33]

Linezolid was the first oxazolidinone antimicrobial approved for clinical use. It inhibits the initiation of protein synthesis by preventing the formation of a ternary complex between tRNA, mRNA, and the ribosome.[34] Unfortunately, approximately two years after its introduction, resistance was reported and its genotype has been mapped to a single point mutation in each of the five 23S ribosomal genes.[12] Despite resistance, some MRSA and VRSA isolates are still susceptible to linezolid. For non-resistant strains, linezolid is still a very useful antibiotic because it is available in oral and intravenous forms. This makes it easier to treat a patient with antibiotics outside of the hospital setting or to continue antibiotic therapy when a patient is released from the hospital.[17, 33]

Tigecycline falls into a new class of antimicrobial agents know as glycyclines. The drug is derived from minocycine (a tetracylcine derivative). The drug is structurally related to the tetracyclines, and shares the same mechanism of action. Although there are numerous similarities between the new glycylcycline and the tetracyclines, the drug is able to evade resistance mechanisms.[33, 35] Studies have shown that tigecycline binds 5-

fold more strongly to the 30S ribosomal subunit, than the tertracyclines. It is believed that its enhanced ability to bind the ribosomal subunit is responsible for its ability to overcome the normal mechanisms of tetracycline resistance.[35]

Daptomycin is a new cyclic lipopeptide antibiotic. The drug works by disrupting membrane potential of the cell wall of various Gram-positive pathogens. The consequences of the rapid depolarization ultimately leads to the arrest of DNA, RNA, and protein synthesis, resulting in cell death.[36] Daptomycin is administered *via* IV once a day, which is superior to the glycopeptides which must be administered several times a day. Additionally, it is nearly impossible to induce bacterial resistance to daptomycin, though some strains of *S. aureus* exposed to long term use of daptomycin have shown reduced susceptibility to the antibiotic. However, at the current time daptomycin is still an effective antibiotic for treating multi-drug resistant *S. aureus*.[33, 36] The only drawback to daptomycin is that it cannot be used for the treatment of lung infections, as pulmonary surfactant inactivates its antibiotic properties.[33]

The Return to Forgotten Medical Treatments for Resistant *S. aureus*.

There are numerous antibiotic therapies, many of which are useless against antibiotic resistant bacteria. To compound this problem many bacteria have biocidal resistance genes. For instance, there are at least 12 biocide resistance genes in staphylococci,[32] which makes it nearly impossible to clean infected wounds. The persistence of MRSA, and the lack of treatment options, caused some hospitals to revert to maggot therapy. In 80 years, resistant strains of bacteria have forced medical practitioners to seek a cure which was once considered obsolete. Maggot therapy works and is not voided by antibiotic resistant bacteria. However, maggot therapy can only be

used for certain types of infections such as open wounds. In the instances where maggot therapy can be used, it completely solves the problem of MRSA/VRSA. Unfortunately, there are many infections that are not candidates for maggot therapy, which proves antibiotics are still a necessary medical staple.[4, 5] If new antibiotics and alternative therapies are developed there must be a strong understanding as to why *S. aureus* has been popularized as a "super bug".

Virulence Factors

The potentially fatal nature of *S. aureus* is exponentially enhanced by its incredible ability to elude medical treatment. At the current time *S. aureus* has developed resistance mechanisms to nearly every class of antibiotics that are supposed to inhibit or kill the bacterium. The question which arises is what causes the need for antibiotic treatment in the first place. The answer is *S. aureus* has developed mechanisms to void immune defenses. In fact, *S. aureus* has a large arsenal of virulence factors that provide the bacteria with optimal infective potential further propelling the need for antibiotic therapy. The majority of these virulence factors can be assigned to three categories: toxins, enzymes and structural. The availability of virulence factors ensures the survival of *S. aureus* in host tissues by allowing it to stick to eukaryotic cell membranes, resist phagocytosis, lyse mammalian cells, invade deep tissues, and circumvent other immunological defense mechanisms.[37] There are numerous virulence factors, but the following paragraphs highlight the ones that are commonly discussed.

The toxins produced by *S. aureus* have many different functions in terms of their modes of action. *S. aureus* is notorious for an extremely serious infection which develops quite rapidly. The disease state known as toxic shock syndrome can ultimately lead to

multi-organ system failure. The main cause of staphylococcal toxic shock syndrome is caused by toxic shock syndrome toxin-1.[38, 39] The remaining cases (approximately 25%), particularly those of the nonmenstural variety, are more commonly associated with other enterotoxins.[40] Toxic shock syndrome was first described in 1978,[40] since then it has been noted that the toxic shock syndrome toxin-1 gene is more prevalent in MRSA verses methicillin-susceptible *S. aureus*.[38]

Staphylococcal food poisoning results from the ingestion of one or more staphylococcal enterotoxins.[39, 41] In addition to enterotoxins; food poisoning is also related to a unique property of *S. aureus*: it can survive in food kept at a higher osmolarity (for preservation) than any other pathogenic bacteria.[26]

Staphylococcal scalded-skin syndrome is caused by toxins produced predominately by bacteriophage group II and some phage group I and III strains of *S. aureus*. In particular, exfoliative toxin type B, produced by bacteriophage group II is the principal causative agent of scalded skin syndrome by *S. aureus*.[42] Staphylococcal scalded-skin syndrome is characterized by the appearance of large blisters and the separation of the epidermis after infection.[40]

S. aureus produces many cytoxic molecules. Four of these molecules are known as hemolysins, categorized as either α, β, γ, or δ.[39, 41] α-Hemolysin and γ-hemolysin capable of lysing eukaryotic cells, especially erythrocytes. β-Toxin is primarily found in animal isolates. γ-Hemolysin and Panton-Valentine Leukocidin are toxins that affect neutorphils and macrophages. δ-Hemolysin is capable of lysing erythrocytes as well as other mammalian cells, such as membrane bound organelles.[41]

Some of the *S. aureus* protective agents that exert enzymatic effects include: coagulase, staphylokinase, hyaluronidase, various lipases, and penicillinase (previously discussed). Coagulase is perhaps one of the most characteristic properties of *S. aureus*.[43] When coagulase associates with prothrombin it can cleave fibrinogen resulting in clot formation, in plasma.[44] The formation of clots is why coagulase belongs to a group of cell surface adhesions know as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). These adhesion factors bind to extracellular matrix components (such as fibrinogen) allowing for rapid colonization.[45]

The majority of *S. aureus* clinical isolates synthesize the plasminogen activator staphylokinase, which contributes to *S. aureus* evasion of host innate immune defenses. Staphylokinase is a potent fibrinolytic agent that forms a complex with plasminogen to generate plasmin activity that preferentially degrades fibrin. *S. aureus* can be protected from phagocytosis *via* fibrin clots (formed by coagulase activity), and when the bacteria can sustain its existence without the aid of a protective disguise, it digests the clots with staphylokinase and spreads to new areas of the body.[31]

Bacterial hyaluronidases are enzymes capable of breaking down hyaluronate and initiate infection at the skin or mucosal surfaces. Hyaluronate is found in many body tissues and fluids of higher organisms such as umbilical cords, synovial fluid, cartilage, brain, muscle, and is a major component of the extracellular matrix especially in soft connective tissue. Fifty percent of the hyaluronate in the body is found in the skin. When hyaluronate exerts its enzymatic effects on hyaluronidase it breaks down into disaccharides. These disaccharides can be transported and metabolized to supply needed nutrients for a pathogen as it replicates and spreads.[46]

Lipase contributes to skin colonization by hydrolyzing human sebum as a nutrient source. Thus, staphylococcal lipase is thought to play an important role in facilitating bacterial colonization in nutrient-limited environments such as the human skin.[43]

There is a unique virulence factor, that is neither a toxin nor an enzyme, and it could only be considered structural, if you think of paint as having structural properties. This pseudo-structural virulence factor is the carotenoid pigment resulting in the characteristic golden yellow color of *S. aureus*. Researchers in San Diego, California have conducted studies to determine whether or not the pigment provides *S. aureus* with any sort of protective properties. Their studies showed that the pigment provides antioxidant properties. Pigment production directly correlated with the bacterium's ability to withstand oxidants and phagocytosis.[47]

The two main virulence factors that are considered structural defenses are protein A, and the capsular polysaccharide. These structures are of particular interest because the glycomimetics currently developed by the Norris group focus on the capsular polysaccharide. However, when testing glycomimetic compounds to determine their ability to disrupt the capsular polysaccharide, care must be taken to avoid unwanted effects by the other structural defense mechanisms, most notably, protein A. This protein binds to antibodies in a non-specific way. Thus, rather than binding to the antigen binding sites of an antibody, protein A bids to the stem. Specifically, protein A binds to the stem, the Fc (Fragment crystallizable) region of immunoglobulin G (IgG).[48, 49] When IgG is bound correctly, it is bound by its Fab (Fragment antigen binding) region.[50] Since, phagocytic cells have receptors for the Fc region, correct IgG binding essential for

phatocytosis to occur. Consequently, protein A functions to render antibodies (tagging *S. aureus*) unrecognizable to phagocytic cells.

The structural defense that is the focus of both the Norris and Fagan groups is the capsular polysaccharide. The presence of an exterior capsule surrounding staphylococci type bacteria was first noted in 1930.[51] More than 90% of *S. aureus* clinical isolates produce capsular polysaccharides, and have been classified into 11 serotypes.[51, 52] Additionally, capsular polysaccharide strains of *S. aureus* can be divided into two further groups: mucoid or nonmucoid. Serotypes 1 and 2 are known as mucoid[2, 51] and are heavily encapsulated.[51] The remaining identified types are nonmucoid and have a thin capsular layer, known as a microcapsule. Microencapsulated strains cannot be visualized by negative type stains such as the commonly used India Ink.[51] The capsular polysaccharides of serotypes 1, 2, 5, and 8 have been purified and their structures have been determined.[52]

The virulence of the capsular polysaccharide is attributed to the fact that it is a physical barrier that prevents phagocytes from recognizing the bacterium. Specifically, the capsular polysaccharide prevents the complement system from executing its protective immunogenic effects. The complement system includes plasma proteins that target pathogens. When these special complement proteins bind to a pathogen, they may either kill the bacterium directly, or they may signal phagocytic cells to the presence of a foreign body. The capsular polysaccharide prevents the complement proteins from attaching to the surface of the bacteria, thus the bacteria float around without any marker indicating the need for phatoytosis.[53] Additionally, emerging evidence shows that *S.*

aureus capsular polysaccharides with a zwitterionic charge motif contribute to (or induce) abscess formation, which serves to enhance the virulent effects. [37]

Most clinical encapsulated isolates of *S. aureus* are of either serotype 5 (CP5) or 8 (CP8) strains.[54] CP5 and CP8 isolates account for approximately 25% and 50%, respectively, of isolates recovered from humans.[2] However, these numbers do fluctuate from study to study. Though the multitude of studies do support the fact that *S. aureus* CP5 and CP8 are responsible for the majority of infections caused in humans.

CP5 and CP8 have similar trisaccharide repeating units composed of *N*-acetyl-mannosaminuronic acid (D-ManAcA), *N*-acetyl-L-fucosamine (L-FucNAc), and *N*-acetyl-D-fucosamine (D-FucNAc) (Figure 1). The structures of CP5 and CP8 are serologically distinct, and this can be attributed to differences in the linkages between the sugars and in the sites of *O*-acetylation (Figure 2).[54, 55]

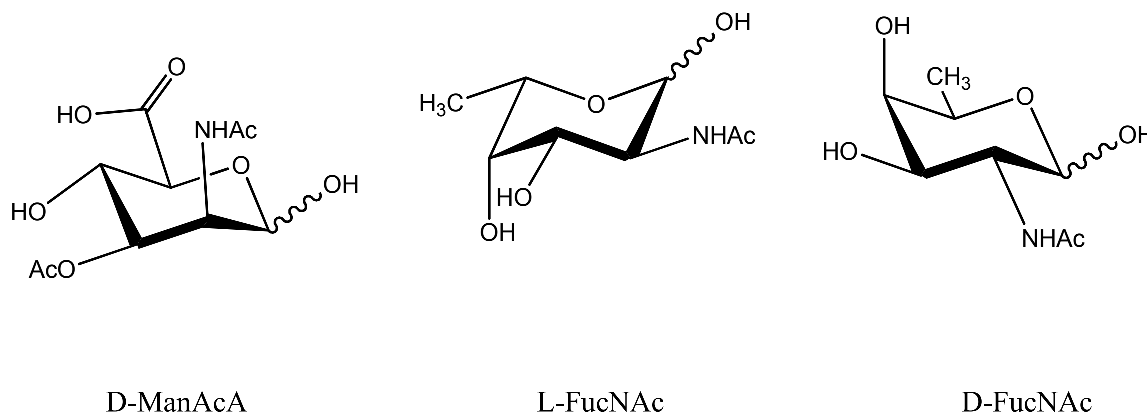


Figure 1: Structures of the three saccharide components of the capsular polysaccharide.

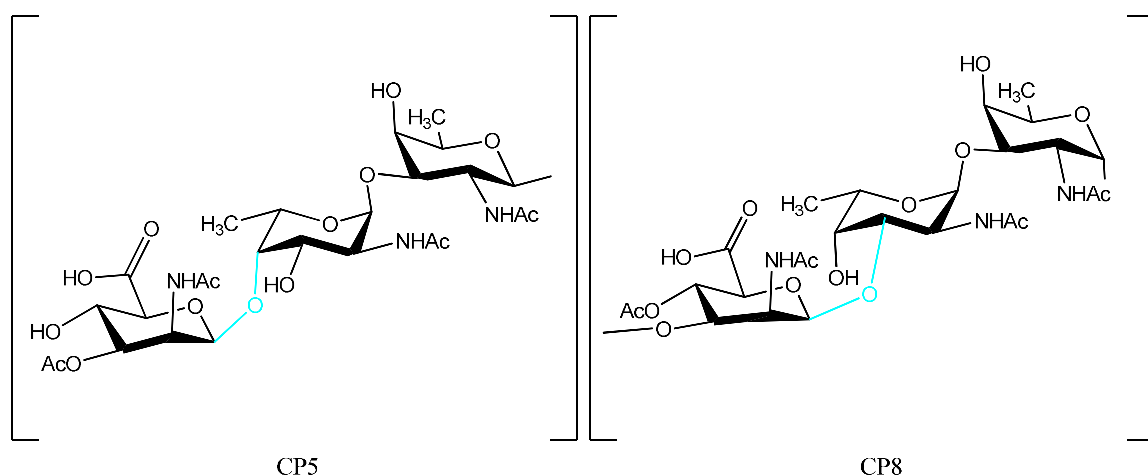


Figure 2: Structures of CP5 and CP8 illustrating the difference in the sites of *O*-acetylation.

Vaccine Targeting the Capsular Polysacharide

A capsular polysaccharide vaccine has been developed as a possible strategy to manage the epidemic nature of MRSA. Normal human serum contains antibodies to the peptidoglycan and other cell wall components present in *S. aureus*. Additionally, some individuals have extremely low levels of antibodies toward CP5 and CP8.[53]

Immunizations containing only purified capsular polysaccharide proved to be non-immunogenic. When the capsular polysaccharide was joined with a carrier protein, an immune response is achieved. Two conjugate vaccines have been created where either CP5 or CP8 is linked to *Pseudomonas aeruginosa* exotoxin A.[56] These vaccines do stimulate an immune response by the body. However, the immune response is short lived and the long-term response is not considered significant when compared to the placebo. However, because there is a short term benefit to the vaccine, it could be used as a possible *S. aureus* preventative measure for surgical patients, or hospitalized patients.[56]

Although, a vaccine targeting CP5 and CP8 *S. aureus* would protect against a majority of disease causing *S. aureus* isolates, there would still be a small percentage of

S. aureus isolates that would not be affected. The remaining clinical isolates produce a cell surface polysaccharide know as 336 polysaccharide. A new vaccine is being developed to target *S. aureus* strains producing the 336 polysaccharide. The hope is to prevent infection caused by a small population of non- type 5 or type 8 *S. aureus* isolates.[57] All *S. aureus* isolates produce a myriad of virulence factors which can lead to devastating consequences if left untreated. Thus, an ideal vaccine would target the capsular polysaccharide and 336 polysaccharide so as to encompass the entire spectrum of *S. aureus* clinical isolates.

Developing Alternative Therapies Targeting *S. aureus*

One possible way to deal with antibiotic resistant *S. aureus* is to prevent production of the capsular polysaccharide. Although, there have been shortcomings with the newly developed vaccine, the capsular polysaccharide is still a promising target for developing therapies against multi-drug resistant *S. aureus*. Recently, the biochemical pathway has been proposed that illustrates the synthesis of CP5 production.[2] Manipulating the proposed pathway is key to developing glycomimetics that could potentially serve to inhibit capsular polysaccharide synthesis. Specifically, carbohydrate mimetics can be developed to mimic the structural and functional properties of the various subunits within the capsular polysaccharide. Additionally, carbohydrate mimetics can be designed to mimic the precursors of the capsular polysaccharide. For example, the three sugars of the capsular polysaccharide are all derived from UDP-*N*-acetyl-D-glucosamine (UDP-D-GlcNAc).[2] Perhaps by altering UDP-D-GlcNAc, the mimic could be incorporated into the biochemical pathway forming the capsular polysaccharide. The result may lead to the cessation of capsular polysaccharide production. Alternatively, the

mimic could successfully progress through the biochemical pathway, resulting in an altered product being incorporated into the capsular polysaccharide. If alternative products are incorporated into the capsular polysaccharide, either by mimicking the capsular polysaccharide sugars or its precursors, there is the possibility that the function of the capsule will be altered, perhaps resulting in decreased virulence of *S. aureus*.

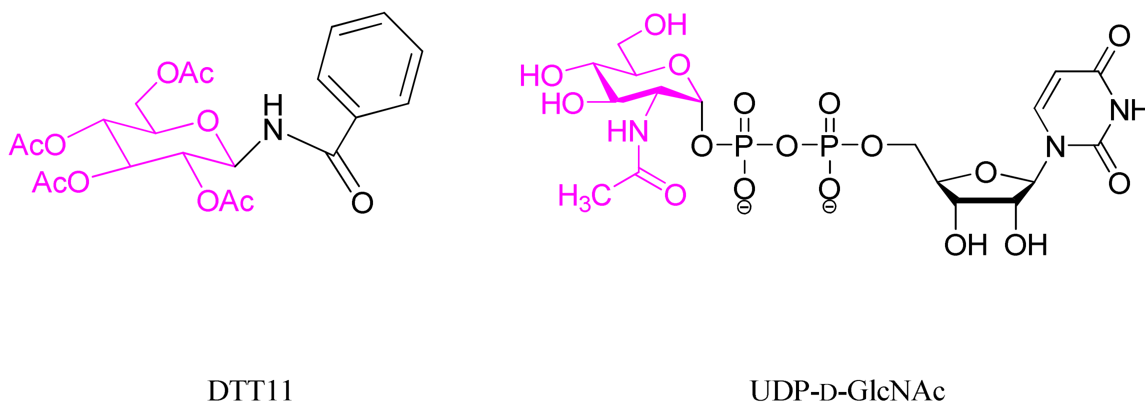
Statement of Problem

Currently, MRSA kills more people in the United States than AIDS.[58, 59] Treatment options are limited due to antibiotic and biocide resistant strains. A novel approach to targeting resistant *S. aureus* is to focus on the capsular polysaccharide, which protects the bacteria from phagocytosis. The Norris group has consistently demonstrated success in synthesizing compounds that structurally resemble carbohydrates associated with the capsular polysaccharide. Unfortunately, in an organic chemistry research laboratory, there are limited resources in terms of testing the biological activity of synthesized compounds. The function of the compound is necessary to determine the success of synthesizing a useful mimetic. The focus of this project will explore the current method used for testing the glycomimetic compounds, and to accesses the results so as to ensure the method provides adequate information regarding the function of a proposed mimetic.

Results:

Testing DTT11

Compound DTT11 is a β-glycosyl amide that is structurally similar to UDP-D-GlcNAc (Figure 3).



DTT11 UDP-D-GlcNAc

Figure 3: A structural comparison of DTT11 and UDP-D-GlcNAc

Two different primary antibodies were used in this experiment involving the effect of compound DTT11 on capsular polysaccharide production: αT5 adsorbed antisera and hybridoma MS.TS.B2.G2.F11. These antibodies were specifically developed to bind to the capsular polysaccharide of Type 5 12602 *S. aureus*. The use of tissue culture treated polyvinyl microtiter plates ensures that the bacteria will stick to the plate and will not be discarded with the wash solution. Therefore, if the primary antibody does bind to the capsular polysaccharide, it also would remain bound after the washing process. If capsular polysaccharide production was interrupted by the presence of compound DTT11, the primary antibody would be unable to successfully bind to the capsular polysaccharide. Thus, the antibody would be discarded with the wash solution. The secondary antibody, anti-mouse polyvalent immunoglobulin peroxidase, will specifically bind to the primary antibody. If there is no primary antibody available for binding, the secondary antibody would be discarded in the washing process. However, if the

secondary antibody is able to successfully bind to the primary antibody, 3,3',5,5'-tetra-methylbenzidine (TMB) would react with the peroxidase and a blue color would result. In this experiment, both of the primary antibodies used were able to successfully bind to the capsular polysaccharide.

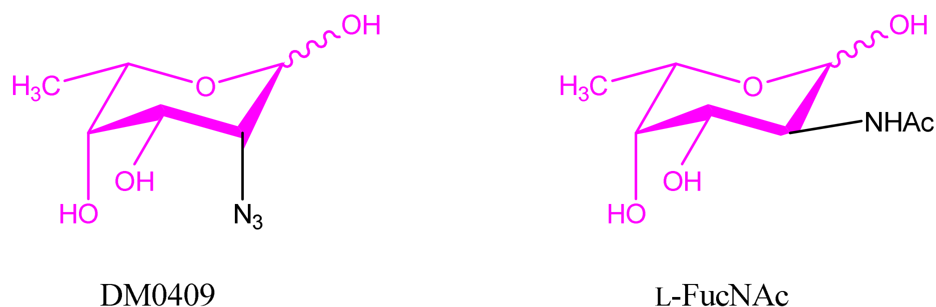
When analyzing the graph for αT5 adsorbed antisera (Figure 4) we notice that the various concentrations of DTT11 and the ethanol control, exhibit the exact same trend. More importantly, the data sets are grouped very close together. Therefore, there is no significant difference in capsular polysaccharide production in the presence of DTT11 or in the presence of ethanol. The same result is evident when analyzing the graph for hybridoma MS.TS.B2.G2.F11 (Figure 5).

It was initially thought that compound DTT11 may inhibit production of the capsular polysaccharide. If this were the case, the absorbance would have decreased in the presence of compound DTT11. Therefore, the data for the various concentrations of DTT11 would fall below the data points for the ethanol control.

Compound DTT11 is protected by several acetyl groups. These groups also affect the solubility of the substance, especially in aqueous environments. Perhaps, compound DTT11 can be altered to remove the acetyl groups, thus making it more like its precursor, UDP-D-GlcNAc.

Testing DM0409

Compound DM0409 is structurally similar to L-FucNAc (Figure 6).



DM0409 L-FucNAc

Figure 6: A structural comparison of DM0409 and L-FucNAc

The different primary antibodies were used in this experiment were hybridoma MS.T8.B2.G2.F11 and hybridoma MS.T8.B2.G2.G2. Again, these antibodies were specifically developed to bind to the capsular polysaccharide of Type 5 12602 *S. aureus*. In this experiment, both of the primary antibodies used were able to bind to the capsular polysaccharide. As seen with compound DTT11, this binding is indicative of mimetic failure. If capsular polysaccharide production had been inhibited by DM0409, the primary antibodies would have been unable to bind to their specific target: the capsular polysaccharide.

When analyzing the graph for hybridoma MS.T8.B2.G2.F11. (Figure 7), it is evident that the various concentrations of DM0409 and the autoclaved DI-H_2O control, exhibit the exact same trend. Again, the data sets are grouped very close together, just as they were in the case of DTT11. Thus, there is no significant difference in capsular polysaccharide production in the presence of DM0409 or in the presence of the control. The same result is evident when analyzing the graph using hybridoma MS.T8.B2.G2.G2 (Figure 8).

Compound DM0409 was designed to very carefully mimic L-FucNAc. Since DM0409 mimics an actual component of the capsular polysaccharide, it was assumed that it would be a more effective inhibitor than compound DTT11. Ultimately the glycomimetics, though structural similar to their target compounds, were unable to function as inhibitors of capsular polysaccharide synthesis.

Discussion:

The inability to inhibit capsular polysaccharide synthesis does not mean the mimetic failed its functional role. For compounds to be considered "mimetics" they must truly mimic the structure and function of a known target.[60] DTT11 is structurally related to UDP-D-GlcNAc. The function of UDP-D-GlcNAc has nothing to do with inhibiting capsular polysaccharide synthesis. Rather, it is supposed to be used as the precursor to forming the sugars which make up the capsular polysaccharide. It is quite possible that DTT11 was able to perform the functions of UDP-D-GlcNAc, but based on the current testing protocol there is no way to determine if the DTT11 was incorporated into the biochemical pathway leading to the synthesis of the capsular polysaccharide.

DM0409 is structurally related to L-FucNAc. The function of L-FucNAc is to simply serve as a structural component of the capsular polysaccharide. As an integral structural component of the capsular polysaccharide it does not have the function of inhibiting capsular polysaccharide synthesis. To characterize DM0409 as a mimetic, it simply must be incorporated into the capsular polysaccharide, in place of L-FucNAc.

The mimetic function cannot be adequately measured by the current method used. Ideally, it would be beneficial if a mimetic could perform the functional role of its target, as well as inhibiting capsular polysaccharide synthesis. However, even if a mimetic is

only able to fulfill the structural and functional roles of its target, it may potentially be able to lessen the anti-phagocytic properties of the capsular polysaccharide. After all, the function of the capsular polysaccharide is related to its structure. If the structure changes, there is a possibility that the function will change as well.

One way to determine if a mimetic compound is incorporated into the capsular polysaccharide, would be to use NMR analysis. However, this suggestion also has its limits. In order to obtain a sample for NMR analysis, the capsular polysaccharide must be separated and purified from the rest of the bacterial components. This is a very time consuming and expensive process.

Another possible way to determine if mimetic compounds are incorporated into the capsule would be to radioactively label them. For example, when researchers wanted to determine the incorporation of tunicamycin into cell membranes, tunicamycin was isotopically labeled. Then cells were incubated in the presence of ^{3}H-tunicamycin. Samples were withdrawn at regular time intervals. The cells were centrifuged, washed and broken by freezing and thawing. The resulting membrane fractions were collected by centrifugation and subsequently washed. The supernant and wash solutions are combined and treated with trichloroacetic acid. The resulting precipitates (representing cytoplasmic material) were collected and separated from the liquid portion (membrane material). Both portions were tested for their radioactivity. The results indicated that tunicamycin was preferentially incorporated into the cell membrane.[61] Perhaps by altering this procedure, it could be used as a basis for testing the ability of mimetic compounds to incorporate into the capsular polysaccharide.

Conclusion:

There are many shortcomings with the experimental procedure used to determine if a proposed mimetic compound inhibits capsular polysaccharide production. Specifically, the "testing" cannot be used to measure the function of glycomimetic compounds that are not explicitly designed to target a molecule that inhibits capsule production. Many of the compounds synthesized in the Norris lab target precursors, the capsule itself, or other structures along the biochemical pathway. In order to accurately and successfully measure the function of proposed mimetic compounds, new testing procedures must be implemented. These new procedures should focus on the incorporation of compounds into the capsular polysaccharide. Alternatively, the Norris group could turn its attention to developing mimetics which target compounds that function to inhibit capsule production, such as tunicamycin.[62]

Experimental:

Developing Primary Antibodies: Antisera

Mice were injected with 10^7 formalin-treated *S. aureus* cells in 500.0 µl of saline. Three types of *S. aureus* were used: Type 5 12602, Type 5 49521 and Type 8. The injections took place on day one (initial immunization), day seven (booster), and day twenty-eight (booster). On day thirty-one, the mouse was given an overdose of anesthetic (Avertin) and its blood was collected by heart puncture. The antibody containing serum was removed by centrifugation and stored at -20 ºC until testing.

Developing Primary Antibodies: Hybridomas

Hybridomas were constructed from mice immunized several times with the following types of *S. aureus*: Type 5 12602, Type 5 49521 and Type 8. After the immunization process, their serum contained positive levels of antibodies directed against one of the three types of *S. aureus* used for immunization. After the mouse's serum had been collected, its spleen was sterilely removed. From the spleen, the mouse's lymphocytes (B-cells) were isolated for the production of hybridomas. After fusion of spleen cells to myeloma cells using 50% polyethylene glycol (PEG), hybridomas were selected with the use of the media supplement hypoxanthine-aminopterin thymidine (HAT). Only cells containing the genetic information of both cell types will survive HAT selection (Figure 8).

The members of the Advanced Immunology class were responsible for fusion of the hybridomas. After HAT selection, wells containing replicating cells could be screened by Enzyme-Linked ImmunoSorbent Assay (ELISA) for the presence of antibod-

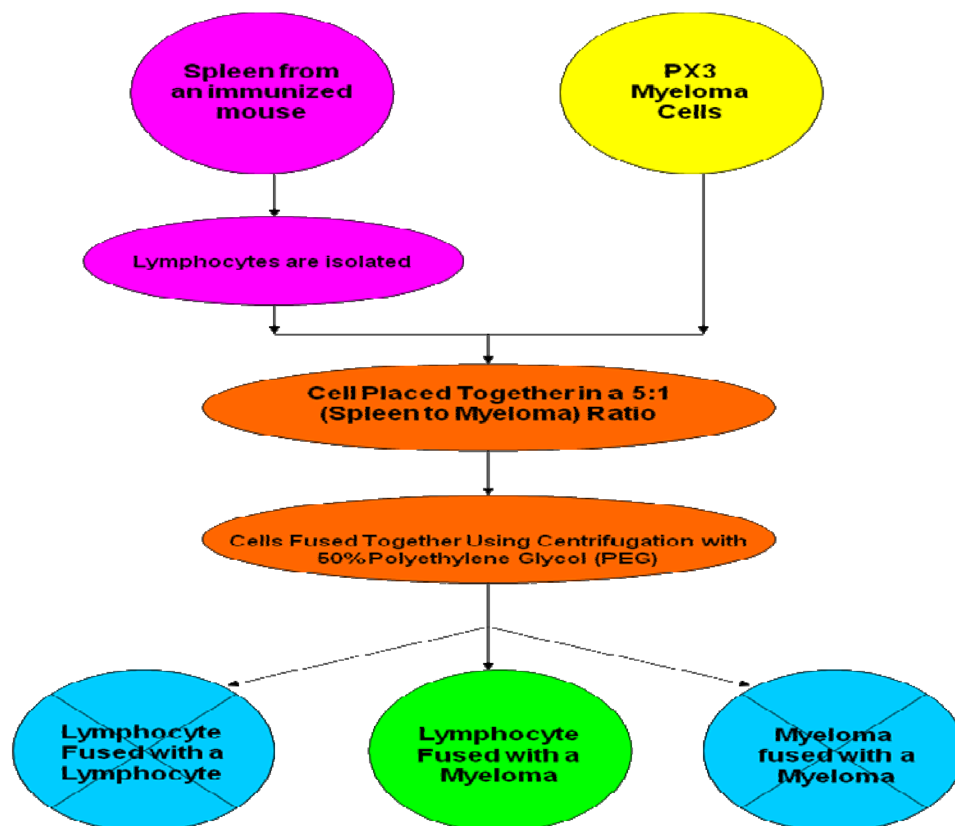


Figure 9: HAT selection

ies directed against the capsular polysaccharide of the three types of *S. aureus* stated above. The ELISA results can be found in Figure 10. The hybridomas that successfully bind to the capsular polysaccharide of *S. aureus* show absorbance values above 0.2 nm. These hybridomas can be used when testing the effect of glycomimetic compounds on the capsular polysaccharide production of *S. aureus*.

Preparation for ELISA:

Four sterile Erlenmeyer flasks containing Columbia broth + 2% NaCl were inoculated with 50.0 µL of type 5 12602 *S. aureus*. Three of the flasks were treated with various concentrations of a proposed glycomimetic compound (1.0 µg/mL, 0.1 µg/mL and 0.01 µg/mL). The fourth flask was treated with 50.0 µL of the solvent used to

dissolve the compound and used as a control. The four flasks were placed on a shaking incubator overnight at 37 °C. The contents of each flask were transferred to individual centrifuge tubes. Then the bacterial solutions were centrifuged at 13,000 x g for 30 minutes. The supernant is discarded, and the bacteria are resuspended *via* vortex. 10.0 mL of phosphate buffered saline (PBS) + 1% Albumin from bovine serum (BSA) is added to each centrifuge tube. The solution is mixed via vortex. The bacterial solutions are again centrifuged at 13,000 x g for 30 minutes. The washing procedure is repeated two more times: once with PBS + 1% BSA and once with PBS. After the PBS wash there is no need to centrifuge the bacteria, as it must remain suspended in the PBS solution for the next step.

The cells are treated with formaldehyde for two reasons: to kill the bacteria, and to preserve the capsule. The bacteria remain in the presence of formaldehyde overnight rotating at 4 °C. Once again, the bacteria are washed twice with PBS + 1% BSA and once with PBS according to the protocol above. Then, the bacteria are treated with trypsin, a protease. This is an imperative step because trypsin destroys protein A, which binds to antibodies nonspecifically (as discussed in the introduction). The bacteria are once again washed. The final bacterial solutions are diluted appropriately until each results in an optical density of 1.0 at 550 nm. The bacterial suspension (100.0 μL) is added to the wells of a tissue culture treated polyvinyl microtiter plate in the following fashion: each plate contains a row of the various concentrations the glycomimetic treated bacteria and one control row. The plates are incubated overnight at 37 °C. Four duplicate plates are made. The plates are centrifuged at 400 x g for 15 minutes.

ELISA:

The wells of the microtiter plates are washed with a solution of PBS + 0.05% Tween-20 (wash buffer). Then 200.0 µL of blocking buffer (PBS + 1% BSA) were added to each well. The plates were placed in the refrigerator where they incubated at 4 °C overnight. The next day, the bacteria treated wells were washed with wash buffer, and the primary antibody was added to each well. The wells of two plates were coated 1:2 serial dilutions of 1 type of primary antibody, and the other two plates were coated with 1:2 serial dilutions of a different primary antibody. (If the primary antibody being used is αT5 adsorbed antisera, the initial dilution is 1:1000. If the primary antibody being used is a hybridoma, the initial dilution is 1:100.) The plates were placed in the refrigerator to incubate overnight at 4 °C.

Following the incubation, each plate was washed three times with wash buffer, and treated with a secondary antibody. Specifically, 100.0 µL of anti-mouse polyvalent immunoglobulin peroxidase in sample buffer (1:1000 dilution) were added to each well. The plates were placed in the incubator at 37 °C for 2 hours. The plates were washed 3 times and then treated with 3,3',5,5'-tetramethylbenzidine. TMB and hydrogen peroxide react with the peroxidase to induce a color change, noted by the development of a blue color. This reaction is halted after 10 minutes by adding 50.0 µL of 2N sulfuric acid to each well. The result is a yellow color. The absorbance of the plates are read at 450 nm.

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Appendix A

ELISA Results

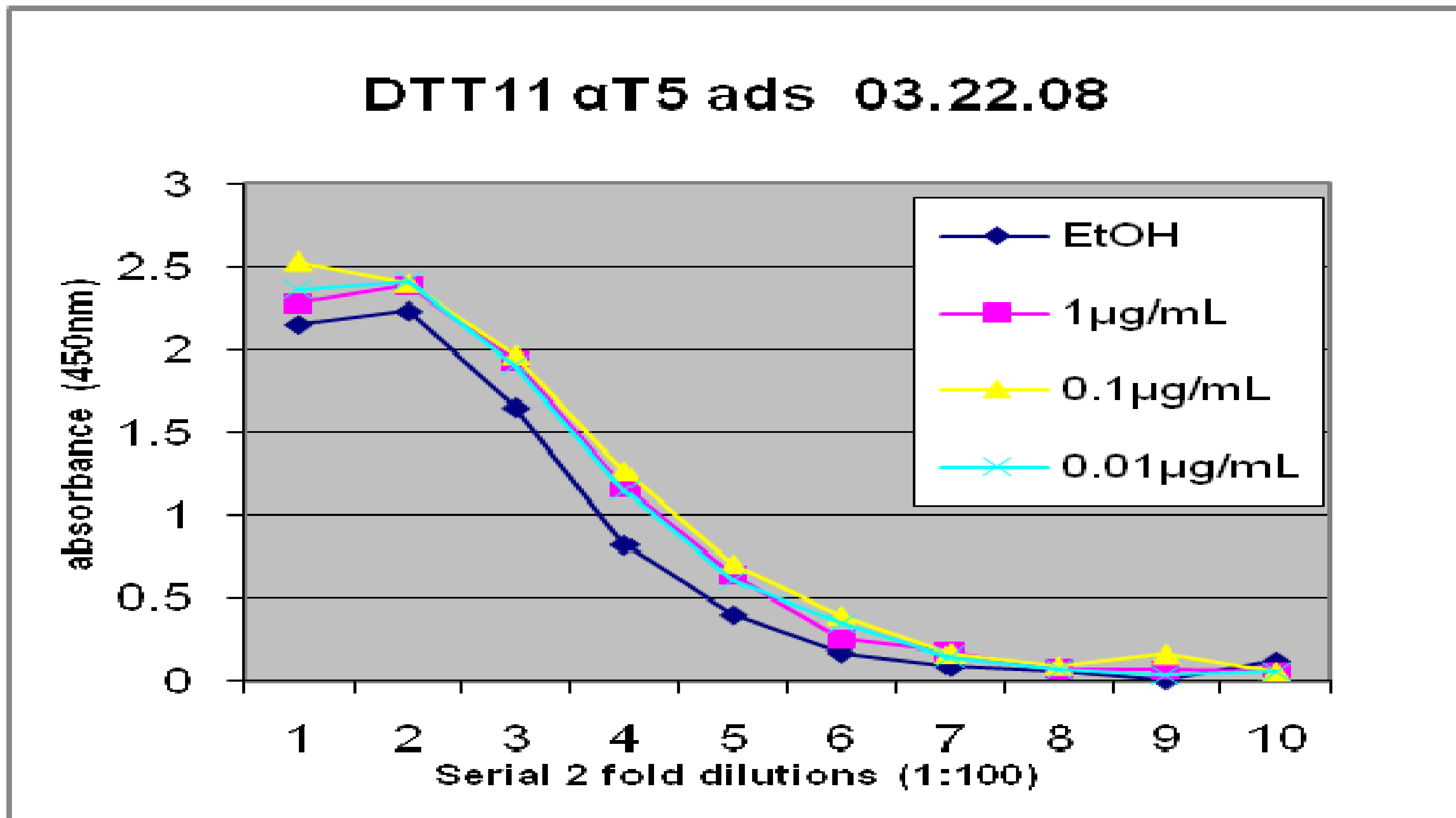

Figure 4: ELISA results using αT5 adsorbed antisera.

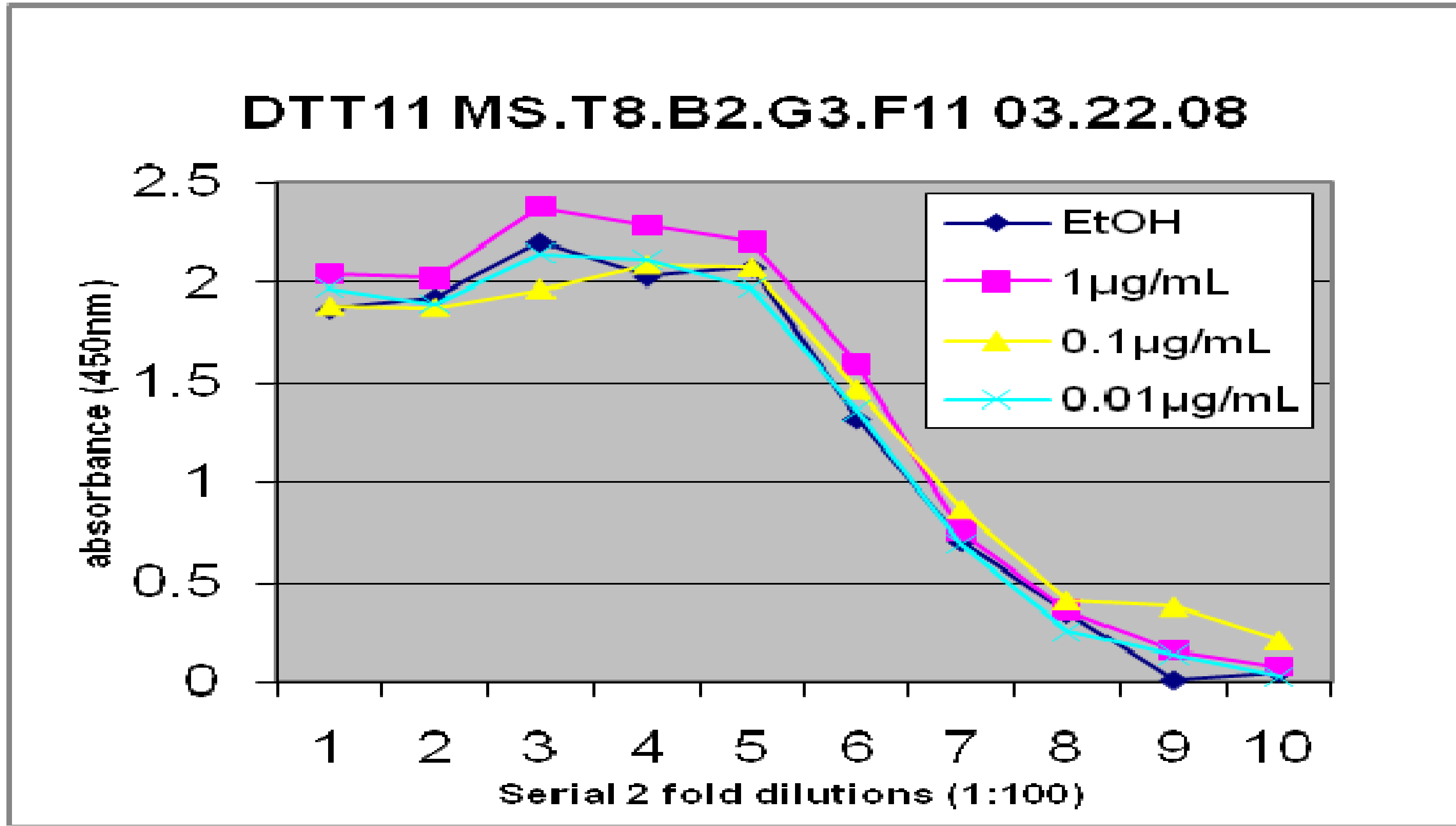

Figure 5: ELISA results using MS.TS.B2.G2.F11.

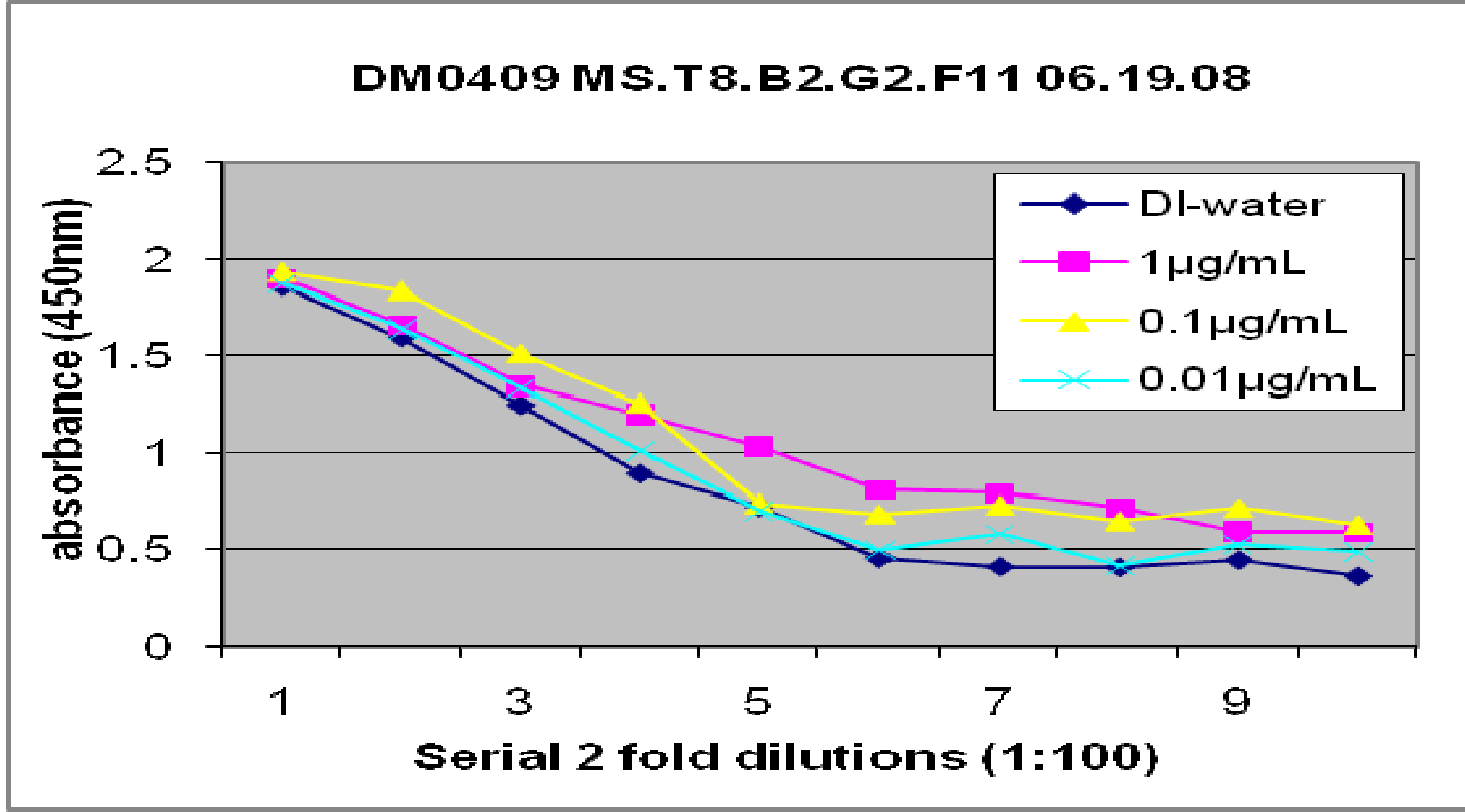

Figure 7: ELISA results using MS.T8.B2.G2.F11.

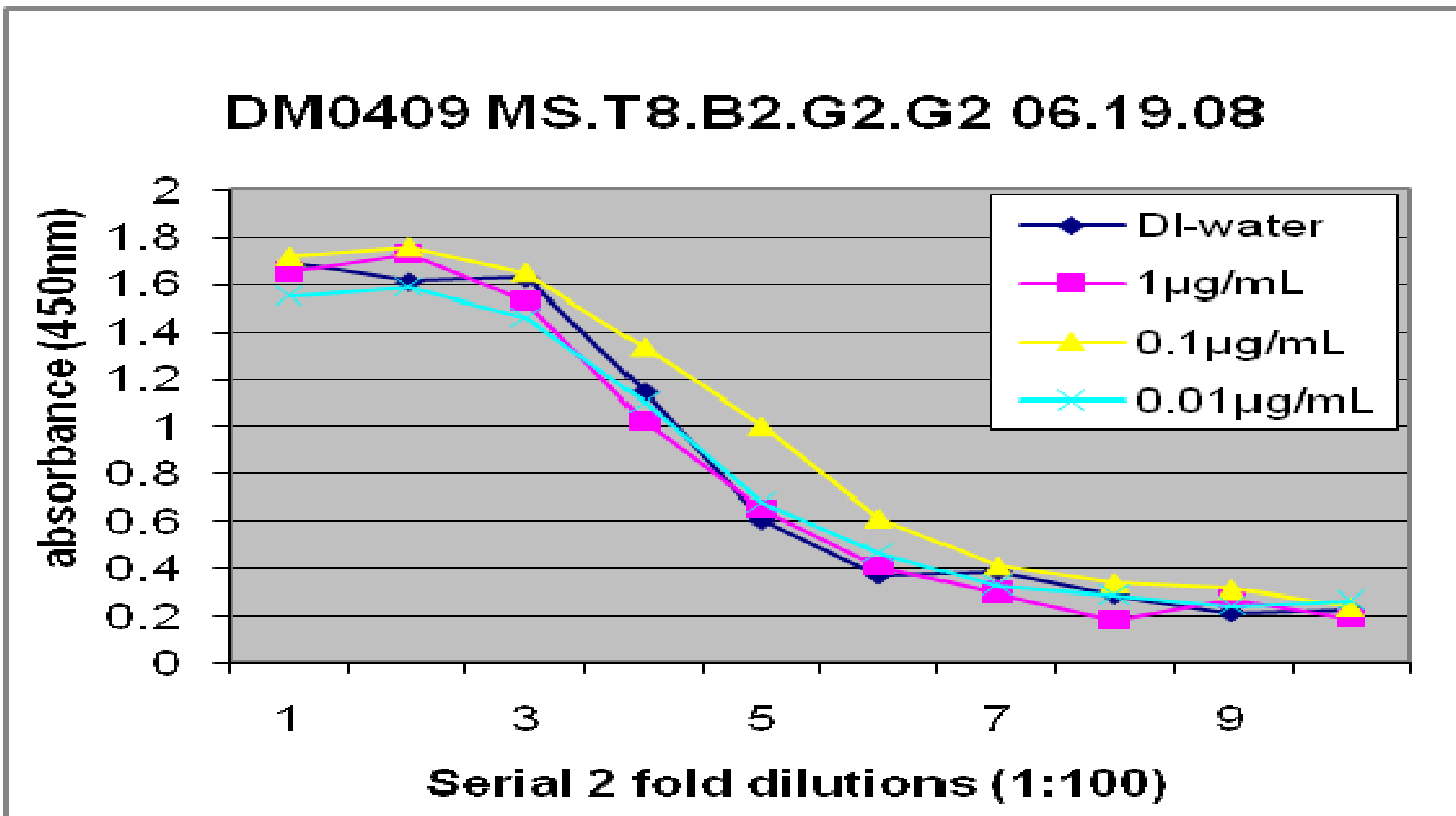

Figure 8: ELISA results using MS.T8.B2.G2.G2.

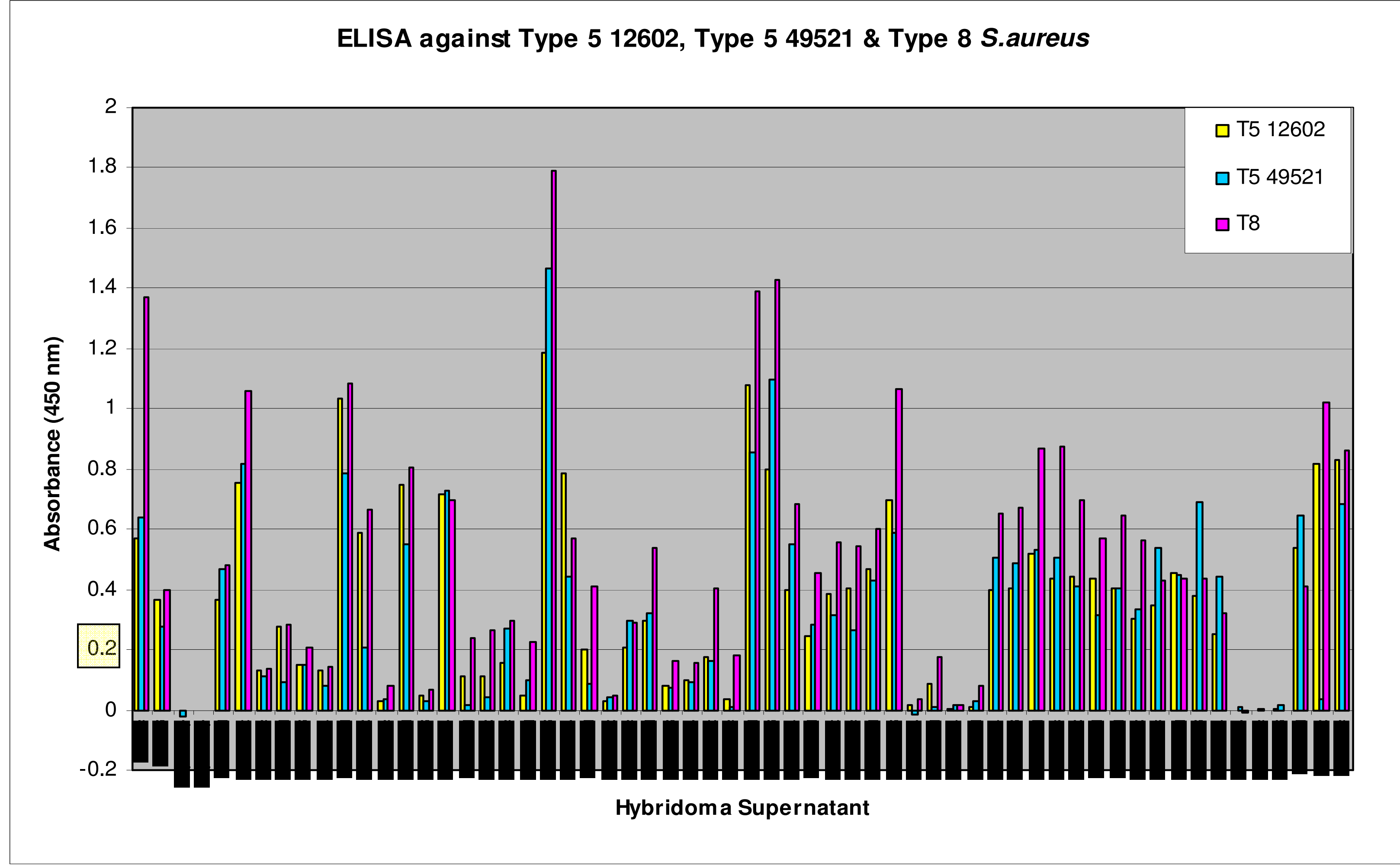

Figure 10: ELISA results for the primary antibodies