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MOLECULAR SUBTYPING OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM THE U.P. COMMUNITY FOR THE PRESENCE OF TOXIN-ENCODING GENES

By

Carol I. Kessel

THESIS

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SIGNATURE APPROVAL FORM

MOLECULAR SUBTYPING OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM THE U.P. COMMUNITY FOR THE PRESENCE OF TOXIN-ENCODING GENES

This thesis by Carol I. Kessel is recommended for approval by the student's Thesis Committee and Department Head in the Department of Biology and by the Assistant Provost of Graduate Education and Research.

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ABSTRACT

MOLECULAR SUBTYPING OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM THE U.P. COMMUNITY FOR THE PRESENCE OF TOXIN-ENCODING GENES

By

Carol I. Kessel

Staphylococcus aureus is the most common cause of human bacterial infections; however, humans can also be asymptomatically colonized with *S. aureus*. Asymptomatic carriers can potentially spread *S. aureus* infection to others. These infections can range from mild to severe. The pathology of a *S. aureus* infection is often dependent on which toxins are expressed and the virulence factors with which they are associated. One goal of this study was to isolate *S. aureus* from healthy, consenting adult volunteers who submitted nasal swabs for culture and qRT-PCR analysis to determine which strains are present in the community. This knowledge could potentially lead to more informed treatment options when *S. aureus* infection is suspected. PCR-based subtyping was utilized to test for the presence of fifteen toxin genes: *lukAB, lukED, pvl, hlgA, hlgC, tst, eta, hla, hlb, hld, sea, seb, sec, sed,* and *see.* Isolates were also tested for the presence of the *mecA* gene, which encodes for methicillin-resistant strains of *S. aureus.* Additionally, nasal swabs were tested for the presence of *Streptococcus pneumoniae* by culture and PCR by students in the Clinical Lab Sciences department. *S. aureus* and *S. pneumoniae* can inhabit the nares in humans, although *in vitro* analysis indicated that *S. pneumoniae* suppresses the growth of *S. aureus*. It was hypothesized that volunteers colonized with *S. pneumoniae* will be less likely to carry *S. aureus.*

Thirty-one strains of *S. aureus* were isolated from the nares of the volunteers, resulting in a colonization rate of 21.9%. Each of the strains were positive for common toxins, such as alpha and delta-hemolysins and hlgA/hlgC. Around half of the strains were positive for the leukocidins LukAB and LukED, and a quarter were positive for toxic shock syndrome toxin. Exfoliative toxin was not discovered in any of the strains. Enterotoxins were discovered to be variant in presence depending on the type of enterotoxin: *sea* had the highest prevalence at 32.26%, whereas *see* was absent from all the isolates.

This study was the first to attempt to identify which genes were present across all six classes of *Staphylococcal* toxins in community-colonized strains of *S. aureus*. We have laid the path toward identifying what kind of *S. aureus* isolates are present in our community. Further analysis using next-generation genome sequencing and *spa* typing will need to be utilized to compare the community strains of *S. aureus* isolated in this study to known disease-causing isolates from the hospital to discern if there is a correlation between the two.

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This thesis is dedicated to my grandfather, Don Kessel. Thank you for always pushing me to be the best student I could possibly be. Thank you for reading to me for countless hours as a child, helping to build the foundation of who I am today. Thank you for being my teacher and mentor, even well into retirement. Finally, thank you for instilling a sense of wonder and passion to never stop learning about the world around me. I love you.

"Those are my golden memories!"

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TABLE OF CONTENTS

List of Tables viii

List of Figures ix

List of Symbols or Abbreviations x

Chapter One: Introduction to *Staphylococcus aureus* and Literature Review 1

Chapter Two: The Interaction between *Staphylococcus aureus* and *Streptococcus pneumoniae* 7

Chapter Three: Common Toxins and Their Associated Genes Expressed by *Staphylococcus aureus* 11

Leukocidins 11

Exfoliative Toxin 13

Enterotoxins 13

Toxic Shock Syndrome Toxin 15

Hemolysins 16

Chapter Four: Experimental Design and Methods 17

Sample Collection & Preparation 17

Identification of *S. aureus* 17

DNA Extraction 19

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) 21

Further Analysis of Positive Strains 23

Antibiotic Sensitivity Testing of *mecA* Positive Strains 24

Chapter Five: Results 25

Volunteer Population 25

Assessment of Toxin Gene Presence within the Isolates 26

Methicillin-Resistant Strains..31

Staphylococcus haemolyticus Strain..34

Mannitol Non-Fermenting Strain 35

Chapter Six: Discussion & Conclusion 37

References 44

Appendix A: IRB Approval Letter 50

Appendix B: Volunteer Consent...52

Appendix C: Volunteer Questionnaire...54

Appendix D: Table of Ct Values for Positive Signal of Toxins Obtained from This Study 57

LIST OF TABLES

Table 1: Control strains of *S. aureus* and *P. aeruginosa* used in this study 20

Table 2: Primer sets used in this experimental design for qRT-PCR 21

Table 3: qRT-PCR reaction mix components used in the experimental design 22

Table 4: Annealing temperatures used for each primer set 22

Table 5: Interpretations of oxacillin minimum inhibitory concentration...........................23

Table 6: Cefoxitin disc zone of inhibition classification for MRSA 23

Table 7: *S. aureus* carriage rates based on age 25

LIST OF FIGURES

Figure 1: Timeline of Prevnar and the start of the increase in *S. aureus* carriage 10

Figure 2: Biochemical tests used to identify isolated strains as *S. aureus* 18

Figure 3: LightCycler 96 analysis of nucA 26

Figure 4: Stacked bar graph showing which strains of *S. aureus* contained which toxins 27

Figure 5: Example of melt curve analyses for *pvl, lukED, lukAB,* and *tst* 28

Figure 6: Example melt curve analyses for *hla, hlb, hld, hlgA, hlgC,* and *eta* 29

Figure 7: Example melt curve analyses for *sea, seb, sec, sed,* and *see* 30

Figure 8: Percentages of each toxin present within the 31 strains of *S. aureus* 31

Figure 9: Biochemical tests used to verify strains were methicillin-resistant 32

Figure 10: Lab analysis printouts of control strains 33

Figure 11: Lab analysis printout of *mecA* community strain..33

Figure 12: Lab analysis printout of *S. haemolyticus* strain 34

Figure 13: Further analysis of the mannitol non-fermenting strain of *S. aureus* 36

LIST OF SYMBOLS OR ABBREVIATIONS

AMPs – antimicrobial peptides
APCs – antigen presenting cells
CA-MRSA – community-associated methicillin-resistant *Staphylococcus aureus*
CDC – Centers for Disease Control and Prevention
CoNS – coagulase-negative staphylococci
ET – exfoliative toxin
Eta – exfoliative toxin A
Etb – exfoliative toxin B
HA-MRSA – hospital-acquired methicillin-resistant *Staphylococcus aureus*
Hla – alpha-hemolysin
Hlb – beta-hemolysin
Hld – delta-hemolysin
HlgA – gamma-hemolysin A
HlgC – gamma-hemolysin C
IRB – Institutional Review Board
LukAB – leukocidin A/B
LukED – leukocidin E/D
MRSA – methicillin-resistant *Staphylococcus aureus*
MSA – mannitol salt agar
NETs – neutrophil extracellular traps
PBP2a – Penicillin Binding Protein 2a
PTSAgs – pyrogenic toxin superantigens
PVL – Panton-Valentine leukocidin
qRT-PCR – quanitatitve real-time polymerase chain reaction
SCC*mec* – staphylococcal cassette chromosome *mec*
Sea – staphylococcal enterotoxin A
Seb – staphylococcal enterotoxin B
Sec – staphylococcal enterotoxin C
Sed – staphylococcal enterotoxin D
See – staphylococcal enterotoxin E
Seg – staphylococcal enterotoxin G
Seh – staphylococcal enterotoxin H
Sei – staphylococcal enterotoxin I
Spa – *Staphylococcus aureus*-specific staphylococcal protein A
TSB – tryptic soy agar
TSS – Toxic Shock Syndrome
Tst – toxic shock syndrome toxin
UPHS-Marquette – Upper Peninsula Health Systems-Marquette
VRSA – vancomycin-resistant *Staphylococcus aureus*

CHAPTER ONE: INTRODUCTION TO *STAPHYLOCOCCUS AUREUS* AND LITERATURE REVIEW

Staphylococcus aureus, an opportunistic pathogenic bacterium, is the most common cause of bacterial infections in humans. *S. aureus* is a Gram-positive coccus found on the skin and inside the nares of mammals. The name is a derivation of two Greek words: "*staphyle,"* which means, "bunch of grapes," and "*kokkos,"* meaning, "berry," named so for its shape and arrangement when observed under a microscope. The bacteria was first discovered in 1880 by a Scottish surgeon by the name of Sir Alexander Ogston, after he described "masses that looked like a bunch of grapes" within the pus from a surgical abscess from a patellar joint [1]. In 1884, another physician named Friedrich Julius Rosenbach distinguished *S. aureus* from another similar species. It was he who designated species names: "*aureus"* for the former, Latin for "gold", and "*albus"* for the latter, meaning "white" but was later changed to "*S. epidermidis"* due to its likelihood of being found on the skin [1].

S. aureus is considered an opportunistic pathogenic bacterium due to its ability to take advantage of damaged cutaneous layers. It can cause infections ranging from mild skin irritation or a simple rash to severe illness including Toxic Shock Syndrome, abscesses, bone infections, and sepsis. These bacteria are capable of wreaking havoc within the human body, primarily by destroying red blood cells through the production of hemolytic toxins [2] and causing sudden drops in blood pressure that can result in death. *S. aureus* is the primary cause of lower respiratory tract and surgical site infections and is the second leading cause of bacteremia, pneumonia, and cardiovascular infections [3].

Furthermore, *S. aureus* can be found in undercooked food or improperly stored food, and when ingested can cause serious cases of food poisoning [4]. These illnesses are caused by a family of toxins called enterotoxins, which are heat-resistant and therefore quite difficult to remove from food once contaminated. Typically, foodborne illness resulting from food contaminated with *S. aureus* is not life-threatening. Although it causes a period of great discomfort, the infection is usually self-limiting within 24-48 hours.

All strains of *S. aureus* contain a thermonuclease enzyme commonly referred to as Nuc, or NucA. It is a known virulence factor in invasive infections caused by *S. aureus* as well as pneumonia caused by other strains of bacteria. Furthermore, NucA allows *S. aureus* to evade neutrophil extracellular traps (NETs). NETs are webs of DNA released by neutrophils in response to bacterial infection. Once neutrophils have become fully functional, the nuclear material becomes condensed and segmented, allowing the cell to essentially sacrifice itself and become a trap of sorts. Bacteria trapped in NETs are more susceptible to lysis. Not only do NETs physically restrict the movement of the bacteria, but the structure is studded with antimicrobial peptides (AMPs). AMPs can disrupt bacteria cell membranes and induce lysis. NucA can degrade NETs in a similar fashion to many other DNases[5]. Degradation of NETs would facilitate *S. aureus* escape from the host immune system.

More recently, a nuclease-like homolog was discovered on the chromosome of *S. aureus*, called Nuc2. A few studies have shown that Nuc2 is active in biochemical assays but does not hold an important role in biofilm development like NucA does [5]. However, in most aspects the two are very similar as they are surface enzymes located on *S. aureus*

with similar biochemical properties when studied *in vivo* and *in vitro* as studied by Kiedrowski et al (2014). The presence of the gene that encodes for NucA is useful as a control to determine that the strain in question is in fact *S. aureus.*

When speaking in terms of bacteria, "colonization rate" refers to the percentage of people who have the bacteria growing as a part of their normal flora; this could be within the nares, on the skin, or in the digestive tract, to name a few examples. In recent years, the rate of colonization of *S. aureus* in the United States was approximately 32.4% [3]. Following colonization, *S. aureus* can also become a part of a person's normal flora. Most people colonized by *S. aureus* are asymptomatic, but this does not mean that these bacteria do not have the potential to be dangerous. In fact, *S. aureus* carriage has a well-documented correlation with infection rates in humans, with approximately 10-40% of people tested upon admission to a hospital for a *S. aureus* infection are found to be nasal carriers of the bacteria [6]. If a host becomes immunosuppressed due to age, stress, disease, or drug therapies for conditions such as organ transplants, asthma, and allergies, *S. aureus* will likely cause an infection.

Historically, antibiotics from the beta-lactam family have always been used to treat *S. aureus* infection. Beta-lactam antibiotics work by inhibiting cell wall synthesis, and are commonly prescribed to treat infections caused by an array of Gram-positive and Gram-negative bacteria. This class of antibiotics received its name due to the beta-lactam ring that makes up their chemical structure. They include two highly-used antibiotic families: penicillins and cephalosporins [7]. *S. aureus* has the capability to produce beta-lactamases, which are enzymes that cleave the beta-lactam ring in the antibiotic structure, rendering it useless [8].

Before penicillin was approved for medical use and used as a treatment option for *S. aureus* infections in 1940, the mortality rate for patients infected by the bacteria was over 80% in a case study done at Boston City Hospital [9]. It was only two years later that the first strain of penicillin-resistant *S. aureus* was observed in a hospital and several resistant strains were found within the community shortly thereafter. Within 20 years, 80% of known *S. aureus* strains were penicillin-resistant [10]. A new antibiotic, methicillin, was introduced in 1959 as a penicillinase-resistant penicillin with hopes for use to be able to kill the newly resistant strains; however, by 1961 certain strains of *S. aureus* had acquired a new gene, *mecA*, and became resistant to methicillin as well. This led to various strains of *S. aureus* to evolve. Overuse of penicillins likely led to the selection of Hospital-Associated Methicillin Resistant-*S. aureus* strains (HA-MRSA) as well as several Community-Acquired Methicillin Resistant-*S. aureus* strains (CA-MRSA).

The primary difference between CA-MRSA strains and HA-MRSA strains is that most CA-MRSA isolates contain the genes for the Panton-Valentine Leukocidin toxin (*pvl*). The majority of CA-MRSA strains are susceptible to non-beta lactam antibiotics, unlike HA-MRSA[10]. HA-MRSA strains tend to be resistant to several classes of antibiotics commonly used to treat various infections. There is also a difference between the two when looking at the type of staphyolococcal cassette chromosome *mec* (SCC*mec*) element that is carried on the genome [11].

Although HA-*S. aureus* infections led to the selection of the first MRSA strains, asymptomatic *S. aureus* carriers are presumably a reservoir for CA-*S. aureus* and can potentially spread CA-MRSA to other people [12]. During a surveillance period from July 2004 to December 2005, 8,987 cases of invasive MRSA infections were reported, and of

those cases, 58.4% were community-onset infections [13]. A second study performed in San Francisco, California found that 90% of MRSA infections in the area during the same time period had community-acquired onset [11]. This has led to MRSA infections becoming the most frequent cause of skin and soft tissue infections that are presented in the ER in the United States to date. Furthermore, approximately 20% of bloodstream infections are caused by *S. aureus.*

As previously stated, methicillin-resistant strains of *S. aureus* are resistant to Penicillin antibiotics (also known as Beta-lactams) due to presence of a gene called *mecA*. The *mecA* gene encodes Penicillin Binding Protein 2a (PBP2a), a transpeptidase that helps build the *S. aureus* cell wall [14]. PBP2a has low binding affinity for Beta-lactams. The presence of PBP2a can therefore allow *S. aureus* to continue building cell walls even in the presence of these antibiotics. These strains of *S. aureus* have become endemic in many United States hospitals, long-term care facilities, and communities [15]. Although many analysts would assume that HA-MRSA strains spread into communities to generate new CA-MRSA strains, data seems to show that the transmittance of the *mecA* gene may in fact be the other way around.

Currently, vancomycin is the drug of choice to treat MRSA infections. This antibiotic has a low chemotherapeutic index, which means that the line between therapeutic levels and toxicity is very narrow. The therapeutic range at trough level (a level drawn right before the first dose of the day is administered) is only between 10.0-20.0 mcg/ml [16], compared to broad-spectrum antibiotics which have a much higher chemotherapeutic index, and are therefore less dangerous to the patient. As of 2010, nine strains of vancomycin-resistant *S. aureus* (VRSA) have been reported in the United

States [17]. The majority of strains of *S. aureus* that are VRSA strains are also resistant to methicillin; however, a strain of VRSA that was methicillin sensitive was discovered in 2015, which begins a new series of questions about how quickly *S. aureus* can develop resistance [18]. As of now, there are few treatment options available to combat VRSA infections.

CHAPTER TWO: THE INTERACTION BETWEEN *STAPHYLOCCOCUS AUREUS* AND *STREPTOCOCCUS PNEUMONIAE*

Like *S. aureus*, *Streptococcus pneumoniae* is an opportunistic pathogen. *S. pneumoniae* is a Gram-positive lancet-shaped diplococcus, is encapsulated by a polysaccharide layer for protection, and is alpha-hemolytic [19]. *S. pneumoniae* can cause a range of diseases including middle ear infections (otis media), sinus infections, meningitis, pneumonia, and bacteremia [20]. The bacterium is most likely to colonize in the upper respiratory tract in humans, especially in children. Colonization rates are highest in childhood, with a peak rate around 55%; they slowly drop off as the child ages until the percentage stabilizes around 8% at the age of 10 and on through adulthood [21]. Pneumococcal infections are extremely dangerous and often deadly if contracted by patients who are immunocompromised, elderly, or children. In the United States, the mortality rate caused by pneumococcal infections is approximately 40,000 people a year [21]. Of those, approximately 18,000 are older adults (>65 years of age), as traditional treatment methods with penicillin-type drugs and other antibiotics are not always effective due to the newly resistant strains of *S. pneumoniae* [22].

Colonization by *S. pneumoniae* in the nares is mostly asymptomatic, again like *S. aureus*. However, *S. pneumoniae* may progress to disease in immunodeficient patients, and colonization by the bacteria leads to person-to-person transfer of different strains to other members of the population. Pneumococcus bacteria are spread from one host to another through droplet-transmission such as coughing, sneezing, or close contact with the bodily fluids of an infected individual [20]. It is recognized that children pose the

greatest risk for person-to-person transfer of these strains to one another; this means the current standard course of action is to focus on prevention of pneumococcal nasal carriage in children.

S. aureus and *S. pneumoniae* compete with one another: that is, they are rarely found co-colonized within the nares of one host. It is hypothesized that this is due to the hydrogen peroxide production by *S. pneumoniae* – it is toxic *in vitro* to other bacteria, including *S. aureus* [23]. The interaction between *S. pneumoniae* and several other common nasal-colonizing bacteria such as *Haemophilus influenzae* and *Moraxella cattarrhalis* have been studied in experiments in which the bacteria were co-cultured. In each case, *S. pneumoniae* had an inhibitory effect on the other bacteria, with the exception of *Neisseria meningitidis*, which appeared to thrive in the presence of the pneumococcal bacteria [21].

A study by Gili Regev-Yochay et al. in 2006 observed a linear relationship between the inoculum level of *S. pneumoniae* and death rate of *S. aureus*. No killing of *S. pneumoniae* by *S. aureus* was observed throughout the course of the experiment. When catalase was added to a co-culture of *S. pneumoniae* and *S. aureus*, death rates of *S. aureus* significantly decreased. This is intriguing because *S. aureus* naturally produces catalase, yet seems to be extremely susceptible to the effects of hydrogen peroxide. This could be due to the inability of *S. aureus* to combat an initial large dose of hydrogen peroxide that is released by *S. pneumoniae* in vitro, although no conclusive data has been published on the exact mechanism to date. One study found that in parallel to a decrease in *S. pneumoniae* colonization rate with age, a simultaneous increase of *S. aureus* carriage is apparent [21].

There is a conventional vaccine that is commercially available aimed to reduce disease caused by *S. pneumoniae* disease: Prevnar. Prevnar is a pneumococcal polysaccharide vaccine that is offered in several different forms, depending on the age of the patient. The first vaccine was licensed in the United States in 2000 and named Prevnar-7 as it protected against seven serotypes of *S. pneumoniae* [24]. In 2010, an updated vaccine was licensed in the United States that protected against an additional six serotypes, and was subsequently named Prevnar-13. Prevnar-13 is now part of the typical vaccination schedule used by pediatric and family medicine clinics in children ages 23 months and younger [25].

The current schedule indicates that Prevnar-13 is to be given concurrently with other childhood vaccines at 2, 4, 6, and between 12-15 months of age [25]. Before the vaccination became routine, the United States saw approximately 13,000 of cases of septicemia, 700 cases of meningitis, and around 200 deaths in children under 5 years of age each year due to *S. pneumoniae.* Since the vaccine has been added to the commercial vaccination schedule for children, these incidences have decreased by approximately 88% [22].

The vaccination is highly effective and an important step towards lowering the rate of preventable mortalities in the high-risk groups outlined above. However, it is hypothesized that by vaccinating to reduce the colonization of *S. pneumoniae,* we are in turn increasing the likelihood of *S. aureus* carriage. This has been observed in a population study from 2012[26] (Figure 1).

It is important to examine which strains of *S. aureus* are colonizing in the nares of the general public as well as which toxins genes these strains are carrying in order to determine their potential pathogenicity.

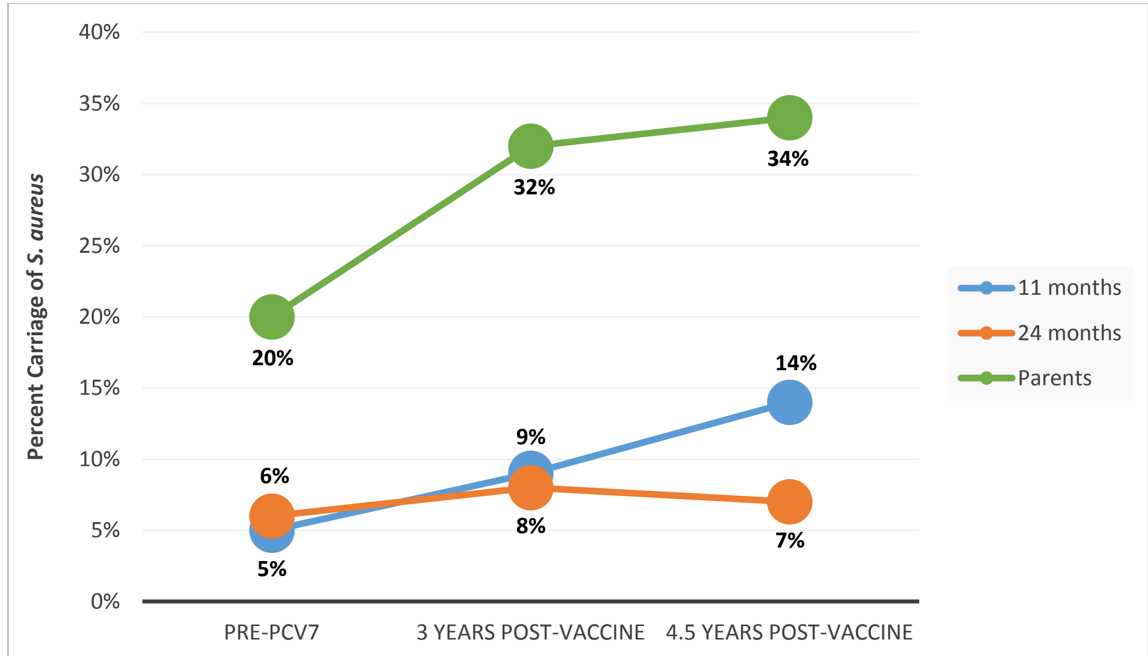


Figure 1: Timeline of Prevnar and the start of the increase in *S. aureus* carriage. This figure shows data from a study by Spijkerman et al (2012) where carriage rates of *S. aureus* were compared with vaccination status of children. Nasal carriage rates were measured before, 3 years after, and 4.5 years after introduction of Prevnar vaccine in children at 11 and 24 months of age, and in parents of 24 month old children. Aside from a 1% decrease in carriage of the 24 month old group, it is clear that there was an increase in *S. aureus* colonization rate after introduction of the vaccine.

CHAPTER THREE: COMMON TOXINS AND THEIR ASSOCIATED GENES EXPRESSED BY *STAPHYLOCOCCUS AUREUS*

S. aureus can cause a wide variety of diseases that range greatly in terms of severity. Examples include simple skin lesions such as pimples/boils, to much more severe including systemic blood and/or bone infections, Toxic Shock, pneumonia, and deep tissue abscesses [2,3,15]. The severity of infection is often dependent on which toxin genes are present, and the virulence factors associated with them. Five classes of toxins were examined in this study: leukocidins, enterotoxins, exfoliative toxin, toxic shock syndrome toxin, and hemolysins.

One of the sub-goals of this thesis research was to determine which types of toxin genes are present in the nasal colonizing *S. aureus* strains found within Upper Peninsula community. By being aware of which strains are present in the community, a better understanding of the virulence factors can be determined, which in turn may aid local physicians in providing more informed treatment for suspected *S. aureus* infections.

Leukocidins

Leukocidins are a type of exotoxin that consist of two synergistic classes of proteins, class S (slow) and class F (fast) [27]. These toxins work by first activating human neutrophils to travel to the infection site and begin the healing process. The toxins then form holes in the cell membranes of the neutrophils [13], which damage the host cell and lead to tissue destruction and cell death. Leukocidins can target several different types of mammalian cells, including monocytes, macrophages, and red blood cells [28]. The

severity of the symptoms resulting from intoxication increases dramatically when these toxins are present. Typically, *S. aureus* infections that are associated with leucocidin producing strains result in furuncles, pneumonia, and some types of diarrhea.

The leukocidins genes targeted in this study were *pvl*, *lukAB*, *lukED, hlgA,* and *hlgC*, specifically. These toxins are commonly found in CA-MRSA strains. Pvl is encoded by two genes in particular, *lukFPV* and *lukS-PV* [27]. These genes code for the proteins, LukFPV and LukSPV, that work synergistically to damage membranes of host cells including erythrocytes and host defense cells [29]. The production of pvl has been linked to severe wound infections, including furuncles, cutaneous abscesses, and necrosis. Pvl production is associated with high morbidity rates, and is known to be an important virulence factor in CA-MRSA, whereas HA-MRSA strains do not typically carry the *pvl* gene [11].

HlgA and HlgC are class S proteins that are encoded as a gamma hemolysin. hlgA constitutes a single open reading frame, but hlgC (and another similar toxin, not tested for in this study, *hlgB*) are co-transcribed. LukED is comprised of two separate genes, *lukE* and *lukD*, and is found on a locus that co-transcribes both S class and F class proteins. LukAB is the most commonly expressed and secreted leukocidin, yet it was only discovered in 2011 [30]. This toxin specifically targets the plasma membrane of the host cell and has a particular affinity for destroying human neutrophils[31]. If the bacterium is phagocytized before lukAB is able to destroy the neutrophils, the toxin facilitates escape from the phagosome by perforating the phagosome via pores until it ruptures, freeing the bacteria [31].

Exfoliative Toxin

Exfoliative toxins (ET) cause Staphylococcal Scalded Skin Syndrome (SSSS), a disease which creates blisters that cover the skin leaving it irritated, red, peeling, and painful [32]. SSSS typically affects infants, and is especially dangerous for this age group due to the comorbid factors that result in the loss of superficial skin layers such as dehydration and secondary infections.

There are two known ET genes, *eta* and *etb,* although for the purposes of this study only *eta* was examined. According to current literature, approximately 80% of exfoliative toxin-encoding *S. aureus* strains have the *eta* gene, rather than *etb* [33], therefore it was determined that searching for *eta* only would suffice for the purposes of this study. ET's are serine proteases around 30 kDa in size that are very specific, in that they only cleave cadherins in superficial layers of the skin, but do not affect the deeper layers of the dermis and subdermis [33]. Cleavage of cadherins results in weepy, raw, red skin; however, as usually only superficial skin layers are affected, this rarely results in a scar. Recent reports show that approximately 3-4% of methicillin-susceptible strains of *S. aureus* carry either the *eta* or the *etb* gene, whereas about 10% of MRSA strains carry *eta.*

Enterotoxins

A second class of toxins is a part of a group called pyrogenic toxin superantigens (PTSAgs), a group that also includes toxic shock syndrome toxin. Enterotoxins effect the digestive system and are commonly the cause of food poisoning related to *S. aureus* contamination in undercooked foods. The enterotoxins found in *S. aureus* are heat stable and cannot be removed from food through heating, making them especially dangerous [2].

Food poisoning that occurs due to *S. aureus* contamination usually consists of nausea and vomiting symptoms that occur within a few hours of ingestion but are self-limiting within a day or two. Staphylococcal food poisoning also typically results in intense intestinal peristalsis, which results in severe abdominal cramping. From 1998 to 2010, 570 confirmed outbreaks caused by *S. aureus* enterotoxins were recorded by the Centers for Disease Control and Prevention (CDC), resulting in 8,015 illnesses and 406 hospitalizations [34].

Eight staphylococcal enterotoxin groups have been recognized to date: *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, and *sei* [34]. Each are small peptide chains of approximately 26 to 29 kDa in length, and are very similar at the amino acid level, with around 51-81% sequence similarity [14,35]. It takes a very small amount of enterotoxin to cause foodborne intoxication – for example, a study has shown that the dose required to produce vomiting was approximately 5-20 μg per animal [35]. In this study, the genes that were specifically examined were *sea, seb, sec, sed,* and *see.* These five genes were chosen specifically because they are the five classified as emetic types, rather than having an "other" classification. There is less certainty about the functions of *seg*, *seh*, and *sei* and what type of illness they cause in mammalian models. The enterotoxins fall into two clades: *sea-see*, for example, fall into clade I, meaning they are closely related to the streptococcal toxins, whereas the latter group are in clade II, and are therefore more distantly related [35].

Toxic Shock Syndrome Toxin

The aforementioned Toxic Shock Syndrome (TSS) is a serious condition that results in a sudden, sharp drop in blood pressure that can result in death [4]. The rapid drop in blood pressure is due to the ability of the TSS to cause host immune cells to release large amounts of potent vasodilating cytokines [13]. TSS can crosslink T-cell receptors to the MHCII receptors on the surface of antigen presenting cells (APCs). This process occurs even in the absence of antigens and causes a massive overstimulation of both APCs and T-cells. This then generates a cytokine storm that results in a fever, rash, shock, and progressive multiple organ failure [36]. Data shows that TSS does not have a greater prevalence within any specific age groups; it is often found in previously healthy, young patients. In the 1980s, a link was recognized between TSS and menstruation/ tampon usage in women [37]. A tampon saturated with blood is a supportive place for rapid growth of bacteria, including *S. aureus*, which is often present in the vagina as normal flora. The insertion and removal of a tampon can cause microscopic tears in the walls of the vaginal canal, allowing the bacteria to enter the bloodstream. Since this discovery, there has been a warning on tampon boxes to caution women against improper use to reduce the risk of TSS.

TSS has been clinically shown to be associated with *S. aureus* strains that express the Toxic Shock Syndrome Toxin (*tst*) [4]. In previous studies, approximately 14% of community strains that were tested were positive for *tst* alone, however the gene seemed to have high comorbidity levels with enterotoxins such as *sea, seb,* and *sec* as well [4]. A study in 1990 determined that a single clone of *S. aureus* accounted for 88% of cases of TSS in the study group [38].

Hemolysins

A final class of toxins called hemolysins will rupture red blood cells within the host. One hemolysin in particular, α-hemolysin (Hla), binds to a receptor that has been a well-studied virulence factor for the past century but was only identified within *S. aureus* in recent years. α-Hemolysin binds to the cell surface and forms a pre-pore as the monomers assemble, later transitioning into a β-barrel transmembrane pore that allows ions (such as K^+ and Ca^{2+}) to enter through an aqueous channel, leading to cell death via lysis [39].

A second type, β-hemolysin (Hlb), does not form pores in the plasma membrane, instead acting by using enzymatic activity to result in hemolysis. Beta-hemolysis has been observed to be highly hemolytic in some animal models, such as sheep, but less hemolytic in other models such as rabbits [40] It has been observed that red blood cells are only lysed after the temperature is lowered, which suggests that the lytic activity is not quite as efficient as α-hemolysin [39]. This phenomenon has been referred to as "hot-cold" hemolysis, and has been studied extensively in sheep blood [41].

As previously mentioned, a third type, γ-hemolysin, belongs to a group of genes that code for both hlgA and *hlgC* as the S (slow) component, or *hlgB* as the F (fast) component. γ-Hemolysin works in a similar fashion to PVL, by allowing water-soluble components to bind to the cell surface as a monomer, then oligomerizing into a prepore, and finally transforming in to a hetero-octamer with four S components and four F components [39]. These molecules then form a β-barrel transmembrane pore that crosses the plasma membrane and leads to lysis by the host cell.

The hemolysins examined at in this study were *hla*, *hlb*, and *hld*.

CHAPTER FOUR: EXPERIMENTAL DESIGN AND METHODS

Sample Collection & Preparation

The first step of the experimental design was to obtain an approved Application for Review of Research Involving Human Subjects from the NMU Institutional Review Board (Appendix A). After approval was secured, four separate volunteer sample collections were set between June 2015 and October 2015. At each collection date, healthy, consenting adult volunteers self-swabbed each nostril after signing a consent and filling out the questionnaire provided (Appendix B). The samples were collected by the author and/or several undergraduates/faculty and were labeled with an accession number. The swabs were streaked on blood agar (Hardy Diagnostics) and then stored in Mueller-Hinton broth tubes (Remel) and labeled with the corresponding accession. Blood agar plates were incubated for 18-24 hours at 37 degrees Celsius before further analysis. Mueller-Hinton broth tubes were incubated for 18-24 hours at 37 degrees Celsius before DNA extraction was performed.

Identification of *S. aureus*

The blood plates were screened for any beta-hemolytic bacteria that looked like it could be *S. aureus*: i.e. round, white-to-golden colonies with entire margins. Strains that appeared to be *S. aureus* were re-streaked for isolation on blood agar, and incubated for another 18-24 hours at 37 degrees Celsius. Once verifying isolated, beta-hemolytic colonies were present, the strains were streaked on Mannitol Salt Agar (VWR Scientific) to test for mannitol fermentation (Figure 2). *S. aureus* is capable of fermenting mannitol,

a type of sugar, which decreases the pH of the media by releasing acetic acid and lactic acid byproducts. A decrease in pH results in the neutral red dye in the media to change color from pink to yellow.

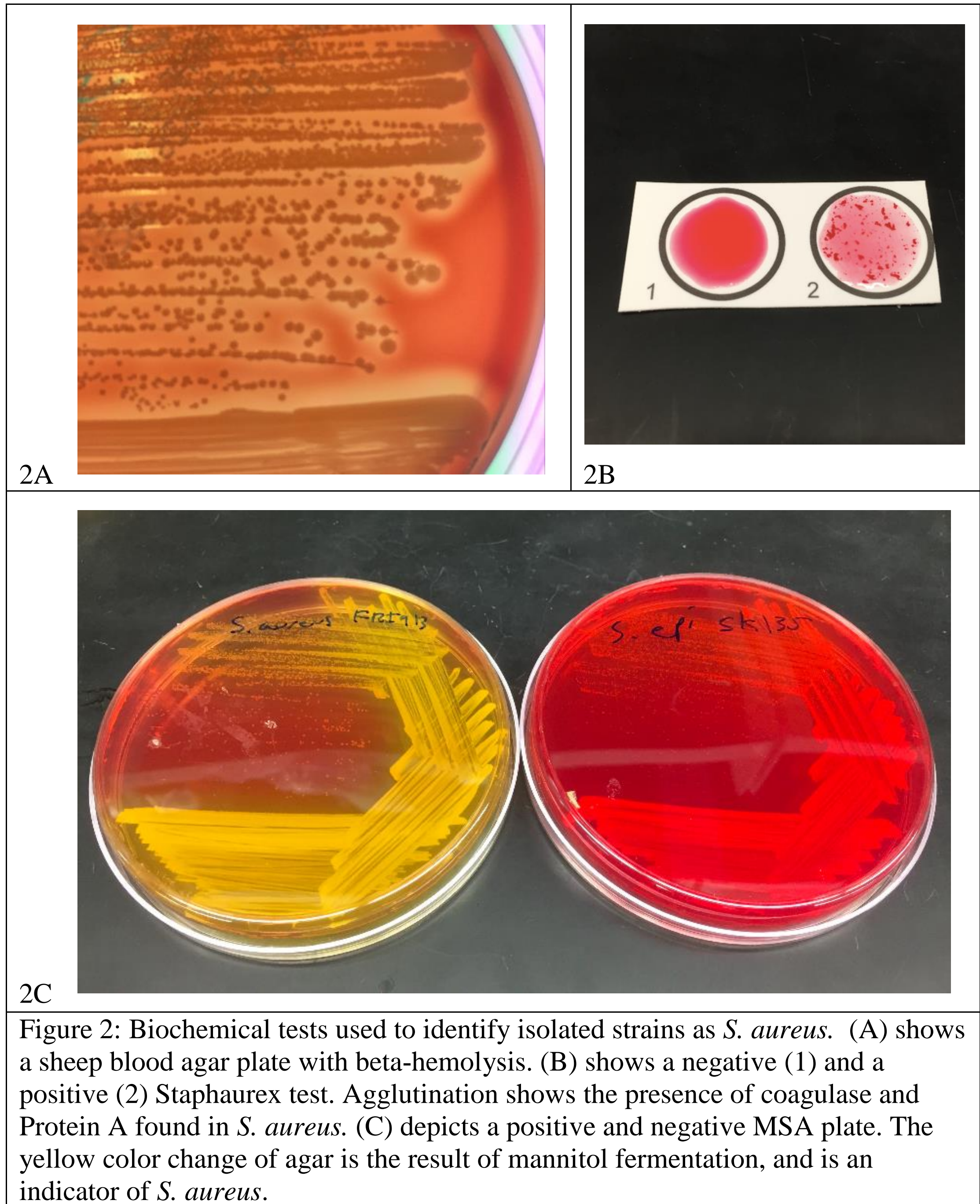


Figure 2: Biochemical tests used to identify isolated strains as *S. aureus.* (A) shows a sheep blood agar plate with beta-hemolysis. (B) shows a negative (1) and a positive (2) Staphaurex test. Agglutination shows the presence of coagulase and Protein A found in *S. aureus.* (C) depicts a positive and negative MSA plate. The yellow color change of agar is the result of mannitol fermentation, and is an indicator of *S. aureus*.

Samples that fermented mannitol were then further tested using Staphaurex (Remel), a biochemical solution that contains latex particles coated with human fibrinogen and IgG that is used to look for the presence coagulase and Protein A, respectively [42]. Coagulase and Protein A are proteins found on the surface of *S. aureus* cells. The positive presence of *S. aureus* causes an agglutination reaction of the latex beads present in the Staphaurex solution. Genomic DNA was extracted from all bacterial isolates that were beta-hemolytic, mannitol fermenting, and Staphaurex positive. Genomic DNA isolation was performed using the QIAamp DNA mini spin kit (Qiagen), essentially as described in the manufactures protocol. Genomic DNA quality and concentration were measured using a NanoDrop spectrophotometer (Thermofisher).

Isolated genomic DNA was tested for the presence of *nucA* using quantitative polymerase chain reaction (qRT-PCR) to verify that the strains were in fact *S. aureus* before testing for toxins. *NucA* is used as the marker for verification of *S. aureus* because it is a thermonuclease enzyme found in all strains of this bacterial species. While the presence of *nucA* is found in a few other staphylococcal species, species-specific primers for *S. aureus* were used to detect the presence of the target bacteria in this study.

DNA Extraction

All mannitol-fermenting, Staphaurex positive strains were used to create 5 ml overnight cultures using tryptic soy agar (TSB) as the medium (Hardy Diagnostics). After incubation overnight at 37 degrees Celcius, the cultures were centrifuged at 4000x gravity for 10 minutes. Following centrifugation, the supernatant was removed, and genomic DNA was extracted from the bacterial cell pellet. The QIAamp DNA mini spin

kit was used per manufacturer's protocol to extract the *S. aureus* DNA, as well as to extract DNA from all positive control strains (Table 1) and from a lab strain of *Pseudomonas aeruginosa* (PAO1) that was used as a negative control throughout the experiment.

Table 1: Control strains of *S. aureus* and *P. aeruginosa* used in this study. The *S. aureus* control strains used in this study were obtained from BEI Resources. MRSA strain BAA 1763 was acquired from the ATCC. PAO1 was obtained from the Dove lab, Boston's Children's Hospital

Strain Name	Positive Control for:	Negative Control for:
A860325	*seb, hla, hld*	N/A
A950206	*sed, hla, hlb, hld, lukED*	N/A
A970675	*hla, hld, eta*	*mecA*
FRI 913	*tst, sea, sec, see*	*mecA*
HFH-30032	*pvl, mecA*	N/A
HFH-30106	*mecA*	*pvl*
HT 20020233	*mecA, seb*	N/A
HT 20020320	*mecA, sea, sed, lukED*	N/A
MNHOCH	*seb*	*mecA*
MN8	*tst*	N/A
USA300	*lukAB, hlgA, hlgC, mecA*	N/A
WKZ-1	*eta, tst*	N/A
1078	*mecA, pvl*	*tst,* enterotoxins
BAA 1763	MRSA strain for plate assays	N/A
FRI913	Methicillin sensitive strain for plate assays	N/A
PAO1	Negative DNA control for all *S. aureus* toxin genes	N/A

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Most of the toxin genes targeted in this study already had qRT-PCR primers that had been previously published (Table 2). The Primerquest Real-Time PCR primer design program was used to design the primers for *lukAB,* as they had not been previously published. Primers were ordered from idtDNA.

Table 2: Primer sets used in this experimental design for qRT-PCR.

Target Gene	Forward Primer	Reverse Primer	Ref.
eta	CTA GTG CAT TTG TTA TTC AA	TGC ATT GAC ACC ATA GTA CT	43
hla	CTG ATT ACT ATC CAA GAA ATT CGA TTG	CTT TCC AGC CTA CTT TTT TAT CAG T	2
hlb	TGA AGA TGG TGG CGT AGC GAT TGT	TCA TGT CCA GCA CCA CAA CGA GAA	44
hld	AAG AAT TTT TAT CTT AAT TAA GGA AGG AGT G	TTA GTG AAT TTG TTC ACT GTG TCG A	2
hlgA	AAT CGG AGG CAG TGG CTC ATT CAA	GGA CCA GTT GGG TCT TGT GCA AAT	45
hlgC	TCG GTG GTA ATT TCC AAT CAG CCC	CGA ATG AAT TCG CTT TGA CGC CC	45
lukAB	CGT GGA GCG TTA ACT GGA AAT A	ACA CCT TTA TGT GAC GTA GAT TGA	This study
lukED	GAA ATG GGG CGT TAC TCA AA	GAA TGG CCA AAT CAT TCG TT	46
mecA	GCA ATC GCT AAA GAA CTA AG	GGG ACC AAC ATA ACC TAA TA	47
nucA	GCG ATT GAT GGT GAT ACG GTT	AGC CAA GCC TTG ACG AAC TAA AGC	48
pvl	GCT CAA CAT ATC ACA CCT GTC AGC GA	AAG TGG GTT GGG TAT AGC CTG AGT	44
sea	AAG TGC CGA TCA ATT TAT GGC TA	CCT GAA CAG TTA CAT TTT TCT TAT TCG T	34
seb	AGA AAA AGG TGA CTG CTC AAG AAT	CGT TTC ATA AGG CGA GTT GTT	34
sec	GCT CAA GAA CTA GAC ATA AAA GCT AGG A	CCT GGT GCA GGC ATC ATA T	34
sed	GAG TTT GAT TCT TCT GAT GGG TCT AA	AAG GTG CTC TGT GGA TAA TGT TTT	34
see	GCT TTG GCG GTA AGG TGC	ATA ACT TAC CGT GGA CCC TTC AGA	34
tst	TTT TTA TCG TAA GCC CTT TGT TG	TAT TAT CGT TTG TAG ATG CTT TTG C	34

The first set of qRT-PCR runs were used to identify that *nucA* was present in all strains and to verify that the strains were all in fact *S. aureus*. The FastStart Essential DNA Green Master kit (Roche) was used following manufacturer's protocol (Table 3), with annealing conditions that differed for each of the specific toxin gene primer sets.

Table 3: qRT-PCR reaction mix components used in the experimental design

DNA	5 µl [≈5ng/µl]
Primer	2 µl [20 µg/µl]
RNase-free H20	3 µl
SYBR Green Mix	10 µl

The PCR cycle conditions that were used consisted of a hot start at 95 degrees Celsius for 600 seconds, followed by 35 cycles of denaturing at 95 degrees Celsius for 10 s, annealing (temperature dependent on primer used – see Table 4) for 15 s, and extension at 72 degrees Celsius for 10 s. This was followed by melt curve analysis. The LightCycler 96 (Roche) and software were used for the qPCR runs and analysis.

Table 4: Annealing temperatures used for each primer set. The annealing temperatures listed below were determined using the suggested annealing temperatures listed on the package insert for each primer set from idtDNA.

Primer Set	**Annealing Temperature**	**Primer Set**	**Annealing Temperature**
eta	45°C	*mecA*	50°C
hla	55°C	*sea*	55°C
hlb	60°C	*seb*	55°C
hld	55°C	*sec*	55°C
hlgA	57°C	*sed*	55°C
hlgC	57°C	*see*	57°C
lukAB	60°C	*tst*	54°C
lukED	54°C	*pvl*	60°C
nucA	55°C		

Further Analysis of *mecA* Positive Strains

If a strain tested positive for the *mecA* gene with qRT-PCR, secondary biochemical testing was used to verify the results were of a resistant phenotype. TSB agar plates (Hardy Diagnostics) supplemented with 4 µg/ml oxacillin (VWR Scientific) were streaked with a positive *mecA* strain (BAA1763) and a methicillin-sensitive strain (FRI913). If growth was present, the result was deemed positive for oxacillin resistance (Table 5). Oxacillin is used because methicillin is not commercially available any longer, and oxacillin holds its reactivity well even during long periods of storage.

Table 5: Interpretations of Oxacillin minimum inhibitory concentration. These values are adapted from the CDC classification of *S. aureus* sensitivities to methicillin.

	Susceptible	Intermediate	Resistant
Staphylococcus aureus	≤ 2 µg/ml	N/A	≤ 4 µg/ml (MRSA)

A second test was performed on Mueller-Hinton agar (Remel) using Cefoxitin discs (Thermofisher) placed using the Kirby-Bauer method. If the zone of inhibition was ≤ 21 mm, the result was deemed positive for Cefoxitin resistance (Table 6). Cefoxitin is most commonly used to detect methicillin resistance because it is a great inducer of *mecA*, and results in clear zones of inhibition that make it easy to analyze results.

Table 6: Cefoxitin disc zone of inhibition classification for MRSA.

	Susceptible	Intermediate	Resistant
Staphylococcus aureus	≤ 25 mm	N/A	≤ 21 mm

Antibiotic Sensitivity Testing of *mecA* Positive Strains

S. aureus strains that contained the *mecA* gene, grew on oxacillin agar, and were cefoxitin resistant were sent to UP Health Systems Marquette to further identify and test for antibiotic sensitivity. A colony of *S. aureus* was suspended in saline and pipetted into a Gram-positive identification/sensitivity plate panel called Pos Combo type 34 (Beckman Coulter). The plate was incubated and read in a Microscan Walkaway 96 plus instrument (Beckman Coulter).

CHAPTER FIVE: RESULTS

Volunteer Population

Of the 141 volunteers, 55 were male (39%) and 86 were female (61%). Of the males, 10 were colonized with *S. aureus* (18.18%) whereas 18 females were colonized (20.93%). Like most studies, these findings suggest that gender does not seem to be a factor in *S. aureus* carriage. As for age, carriage rates do not seem to be statistically significant (Table 7).

Table 7: *S. aureus* carriage rates based on age. Of the 141 total volunteers sampled, the age brackets and carriage rates are listed below to show rates of colonization.

Age	**Number of volunteers**	**Number of positive isolates**	**Colonization Rate Percentage**
18-30 years	87	17	19.54%
31-40 years	15	6	40.00%
41-49 years	6	1	16.66%
50-59 years	16	3	18.75%
60-69 years	7	0	0.00%
75+ years	10	1	10.00%

Typically, bacterial colonization rate studies do not find a correlation between age and carriage rate. This is because bacteria that colonize in healthy noses are just looking for a host that is well enough to aid in the spreading of the bacteria, so therefore age is not typically a factor.

Assessment of Toxin Gene Presence within the Isolates

There were 31 total strains collected and identified as *S. aureus* after the four collections. Each of the strains were positive for *nucA* as determined by two replicates of qPCR using USA300, WKZ-1, and FRI 913 as positive controls (all positive controls contain the *nucA* gene, as they are all *S. aureus* species), and PAO1 as the negative control. Positive signals were seen between 11-16 cycles, also known as the Ct value (Figure 3). A Ct value just refers to the cycle at which fluorescence starts to increase exponentially. Melt curves analysis was used to assess that only the product of interest was amplified, as seen by a single peak around one melting temperature point: multiple peaks would indicate contamination or non-specific amplification.

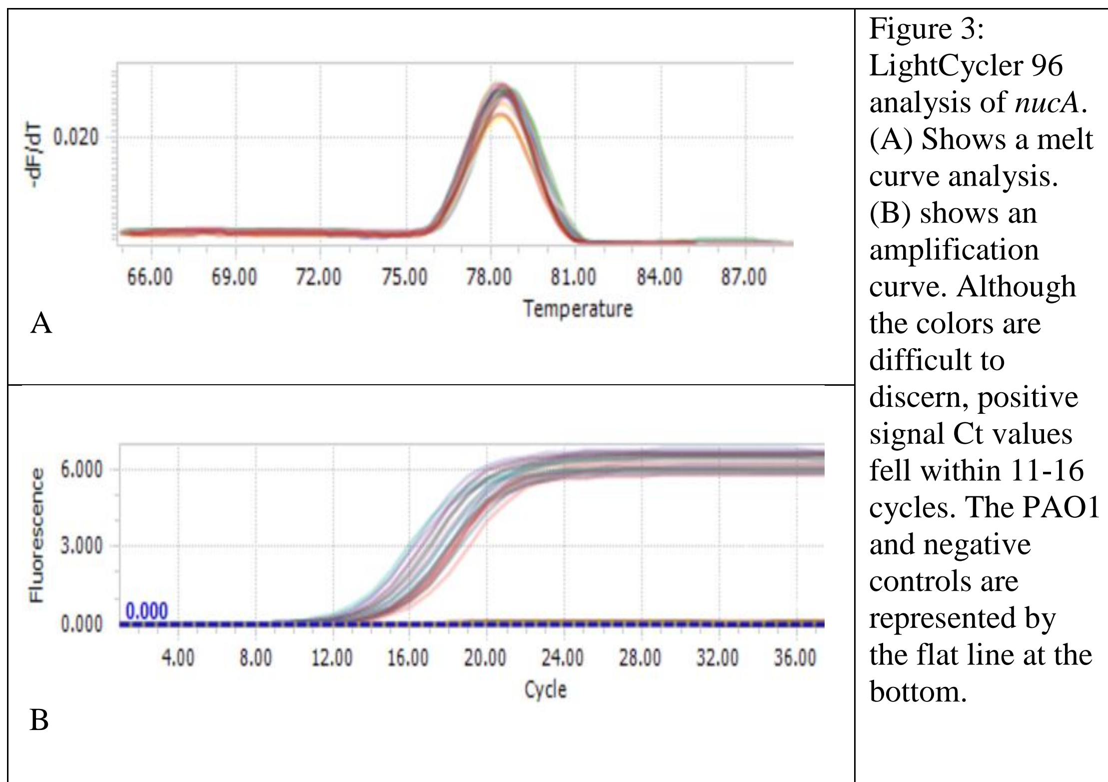


Figure 3: LightCycler 96 analysis of *nucA*. (A) Shows a melt curve analysis. (B) shows an amplification curve. Although the colors are difficult to discern, positive signal Ct values fell within 11-16 cycles. The PAO1 and negative controls are represented by the flat line at the bottom.

Of the 16 toxin genes tested for in the study, 13 were present within the community-found strains (Figure 4). Three of the toxin genes (*pvl, eta,* and *see*) were not found within any of the collected strains.

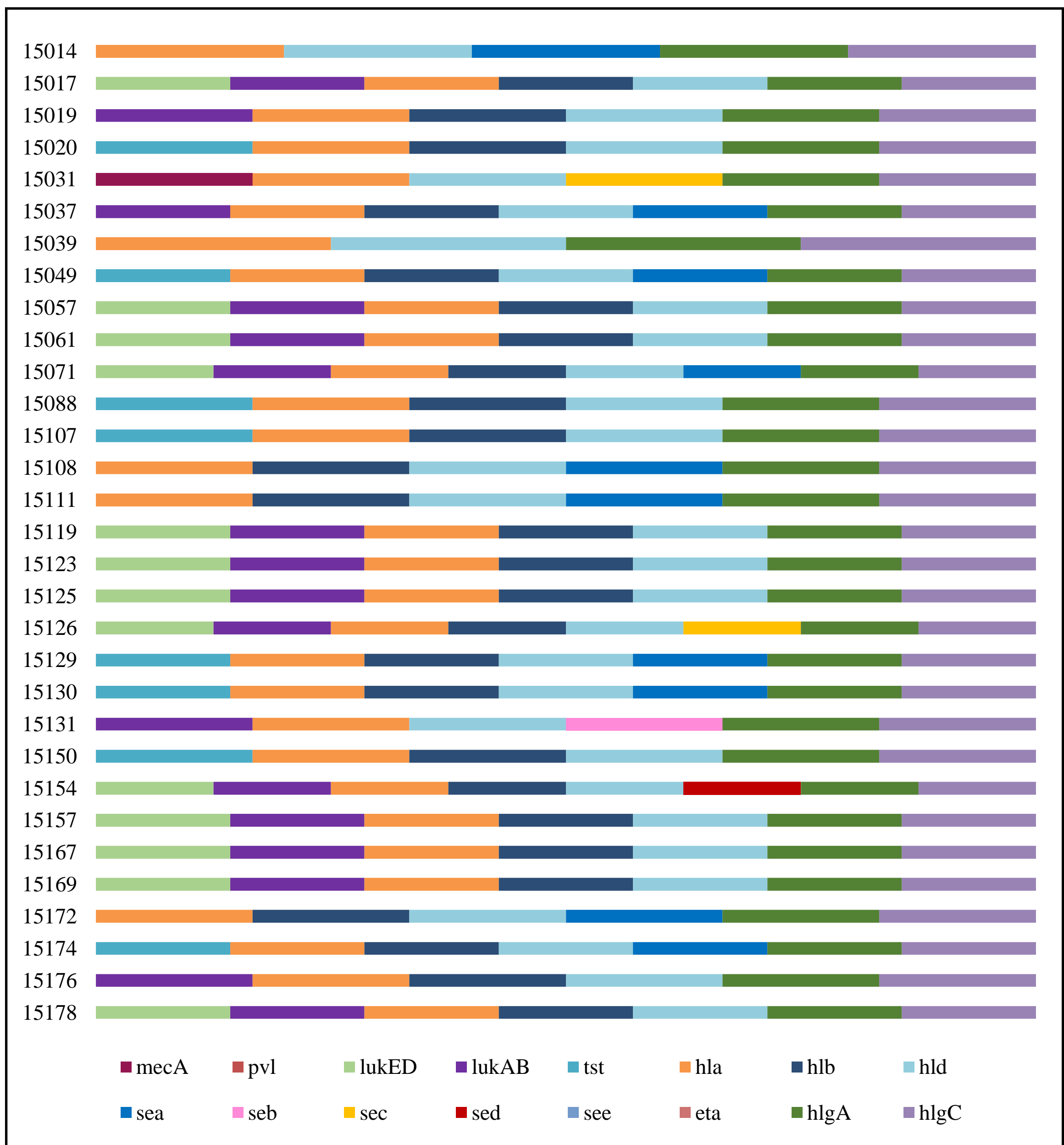


Figure 4: Stacked bar graph showing which strains of *S. aureus* contained which toxins. Some toxins were represented in higher numbers than others, with some toxins (i.e. eta) not represented within the strains at all. Note that the length of the bars is arbitrary, and the bar colors depict the presence of a particular toxin.

The leukocidins group had varying results. Zero of the 31 isolated *S. aureus* strains tested positive for *pvl*, therefore none contained Panton-Valentine leukocidin (Figure 5A). As for *lukED*, 13 of 31 (41.94%) were positive (Figure 5B). The presence of

lukAB was even higher, with 17 of 31 (54.84%) strains positive for the gene (Figure 5C). Furthermore, toxic shock syndrome toxin was present in 25.81% of the strains, with 8 of 31 containing the *tst* gene (Figure 5D).

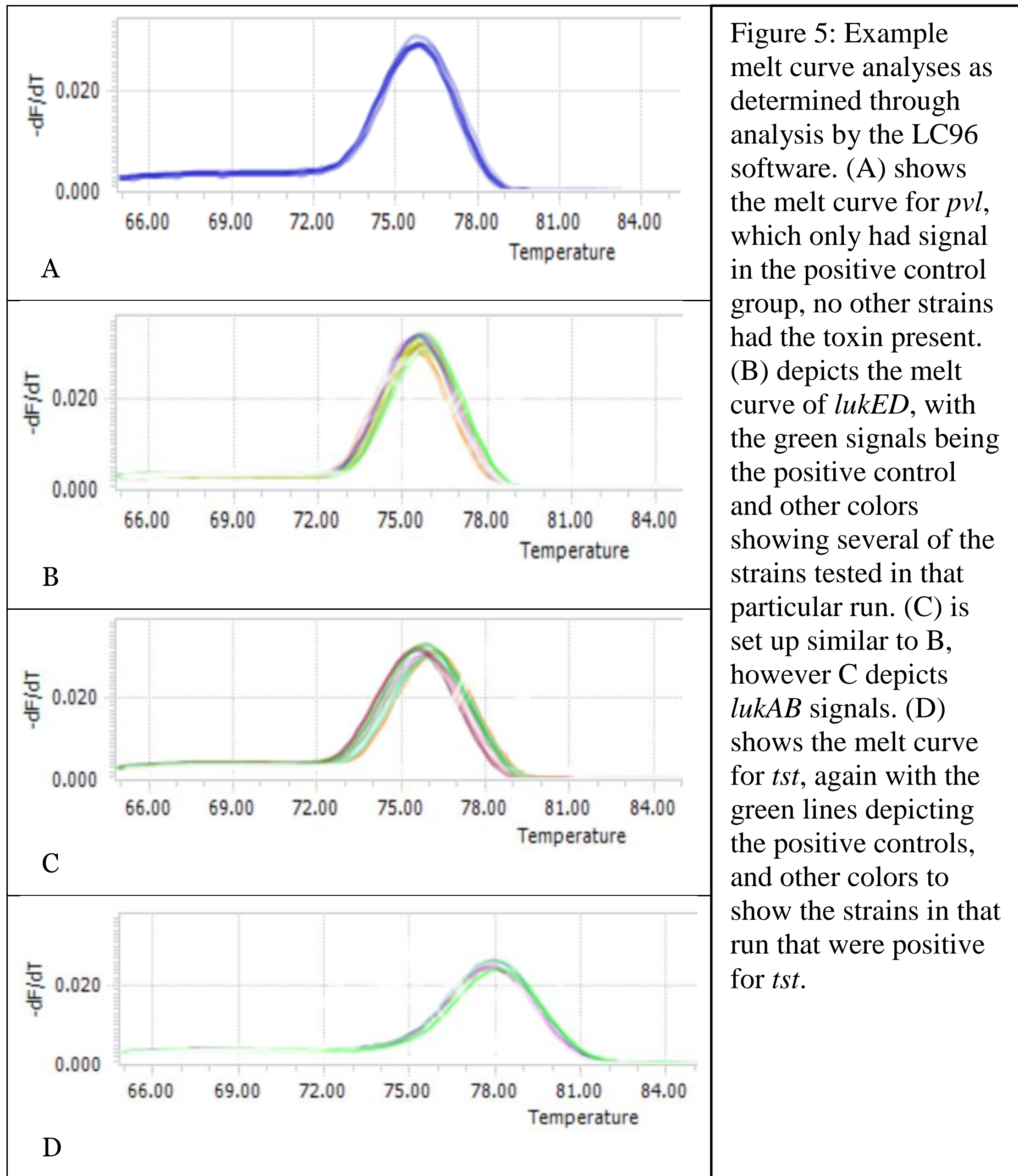


Figure 5: Example melt curve analyses as determined through analysis by the LC96 software. (A) shows the melt curve for *pvl*, which only had signal in the positive control group, no other strains had the toxin present. (B) depicts the melt curve of *lukED*, with the green signals being the positive control and other colors showing several of the strains tested in that particular run. (C) is set up similar to B, however C depicts *lukAB* signals. (D) shows the melt curve for *tst*, again with the green lines depicting the positive controls, and other colors to show the strains in that run that were positive for *tst*.

The hemolysins were, as expected, highly present within the strains. Both *hla* and *hld* were present in 100% of the strains (Figures 6A and 6C), whereas *hlb* was slightly lower, at 27 of 31 (87.10%) strains (Figure 6B). *hlgA* and *hlgC* (Figures 6D and 6E) were

both present in 100% of the strains as well. *eta*, unsurprisingly, was not present in any of the strains (Figure 6F).

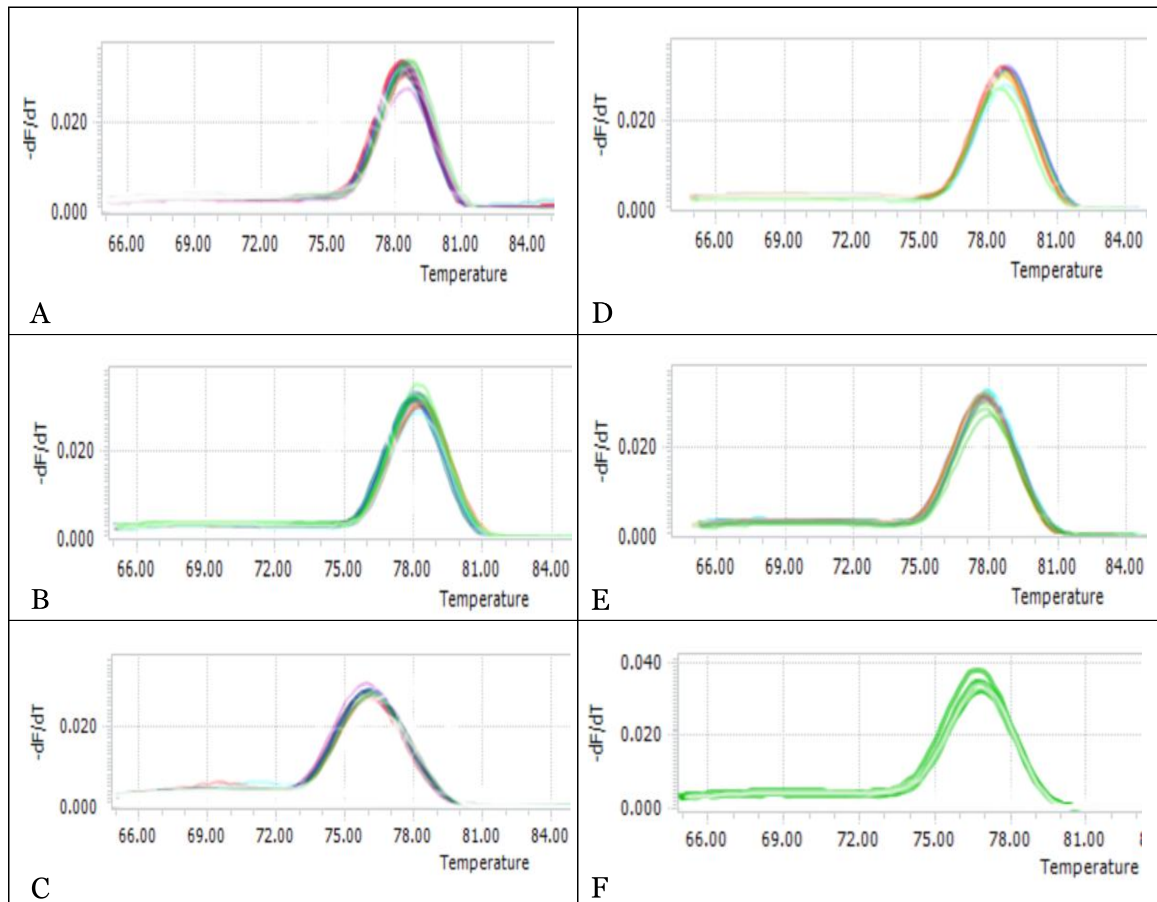


Figure 6: Example melt curve analyses as determined through analysis by the LC96 software. (A) shows the melt curve obtained for *hla*, a hemolysin present in every strain of *S. aureus*. (B) depicts the melt curve for *hlb*, which is a hemolysin found in slightly fewer percent of community strains. (C) depicts *hld* presence, which is 100%, similar to *hla*. (D) shows the melt curve obtained in one of the runs for *hlgA*, which is again found in every strain of *S. aureus*. (E) shows one of the qPCR runs for *hlgC*, also present in 100% of *S. aureus* strains. (F) shows the melt curve analysis for *eta*, which was not present in any toxin. The green shown is the signal by the positive control used, WKZ-1.

The enterotoxins had the highest variation of levels of presence within the strains. The highest prevalence belonged to *sea* with 10 of 31 (32.26%) of strains being positive for the gene (Figure 7A). *Seb* had only 1 of 31 (3.23%) present (Figure 7B), and *sec* had

2 of 31 (6.45%) positive for the gene (Figure 7C). *Sed* was present in 1 of the 31 strains (3.23%) (Figure 7D), but *see* was not present in any of the strains (0 of 31) (Figure 7E).

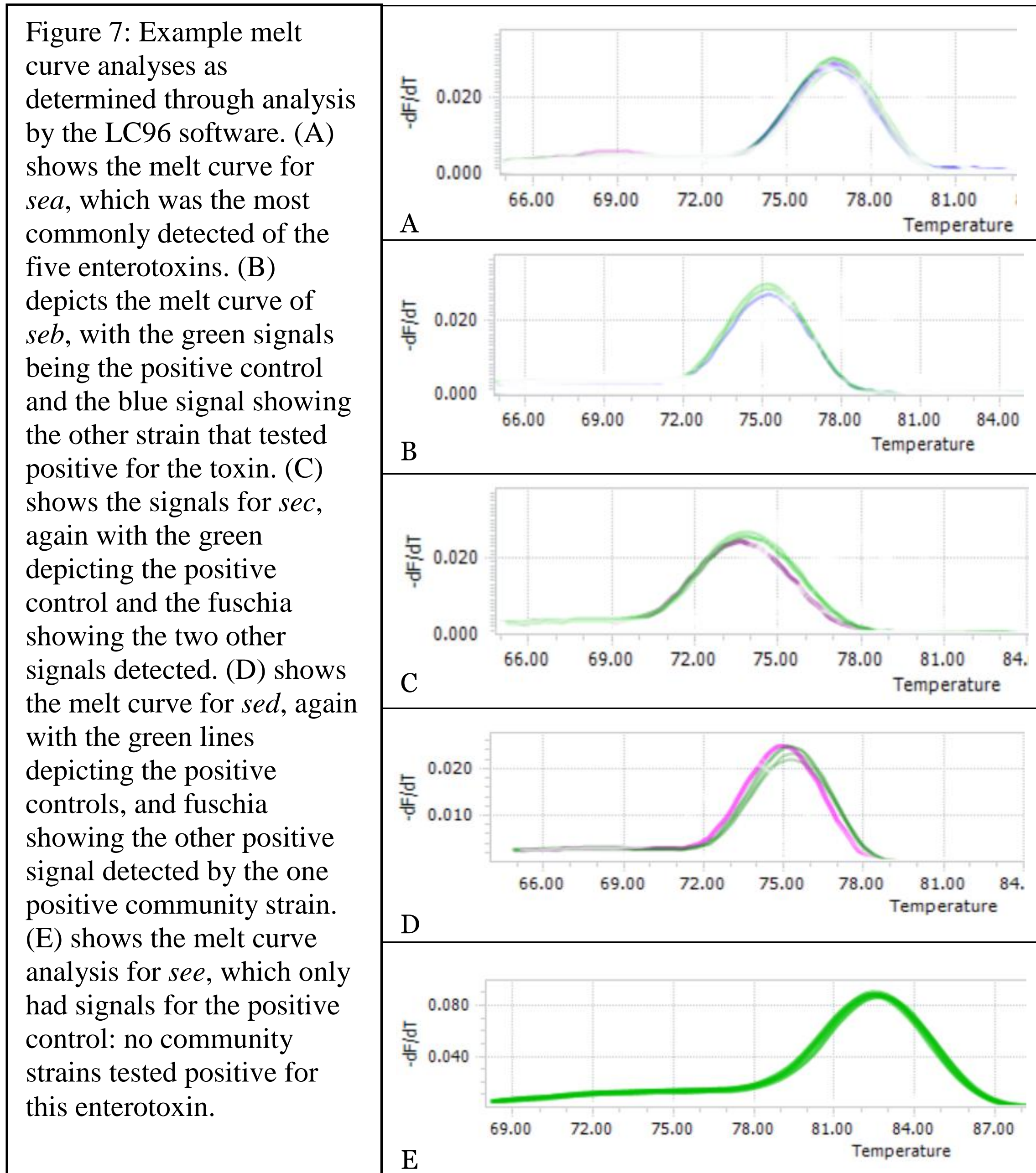


Figure 7: Example melt curve analyses as determined through analysis by the LC96 software. (A) shows the melt curve for *sea*, which was the most commonly detected of the five enterotoxins. (B) depicts the melt curve of *seb*, with the green signals being the positive control and the blue signal showing the other strain that tested positive for the toxin. (C) shows the signals for *sec*, again with the green depicting the positive control and the fuschia showing the two other signals detected. (D) shows the melt curve for *sed*, again with the green lines depicting the positive controls, and fuschia showing the other positive signal detected by the one positive community strain. (E) shows the melt curve analysis for *see*, which only had signals for the positive control: no community strains tested positive for this enterotoxin.

Figure 8 shows a breakdown of what percentages of each toxin was present within the 31 total strains of *S. aureus*.

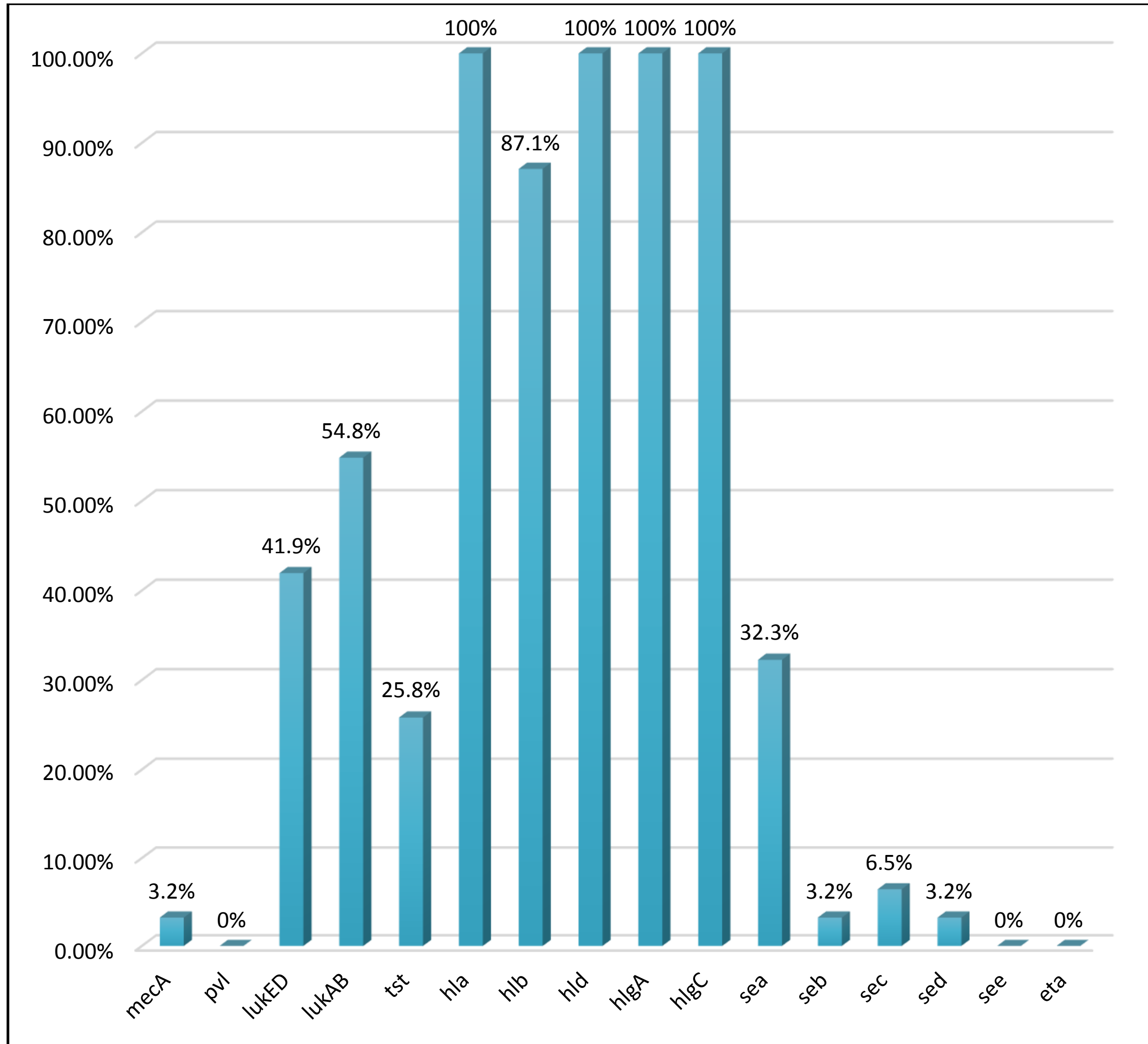


Figure 8: Percentages of each toxin gene and *mecA* present within the 31 strains of *S. aureus*. Percentages are shown above each bar.

Methicillin-Resistant Strains

Of the 31 strains of *S. aureus* isolated from the nares of volunteers within the community, only one strain (15031) was positive for *mecA*. This strain was further tested using Cefoxitin discs and Oxacillin (Figure 9). After testing positive with growth present

on the Oxacillin field plate as well as growth within the parameters set in Table 5 (see previous chapter), it was determined that strain 15031 was likely methicillin-resistant *S. aureus*, and was sent to the UPHS-Marquette microbiology laboratory for further testing.

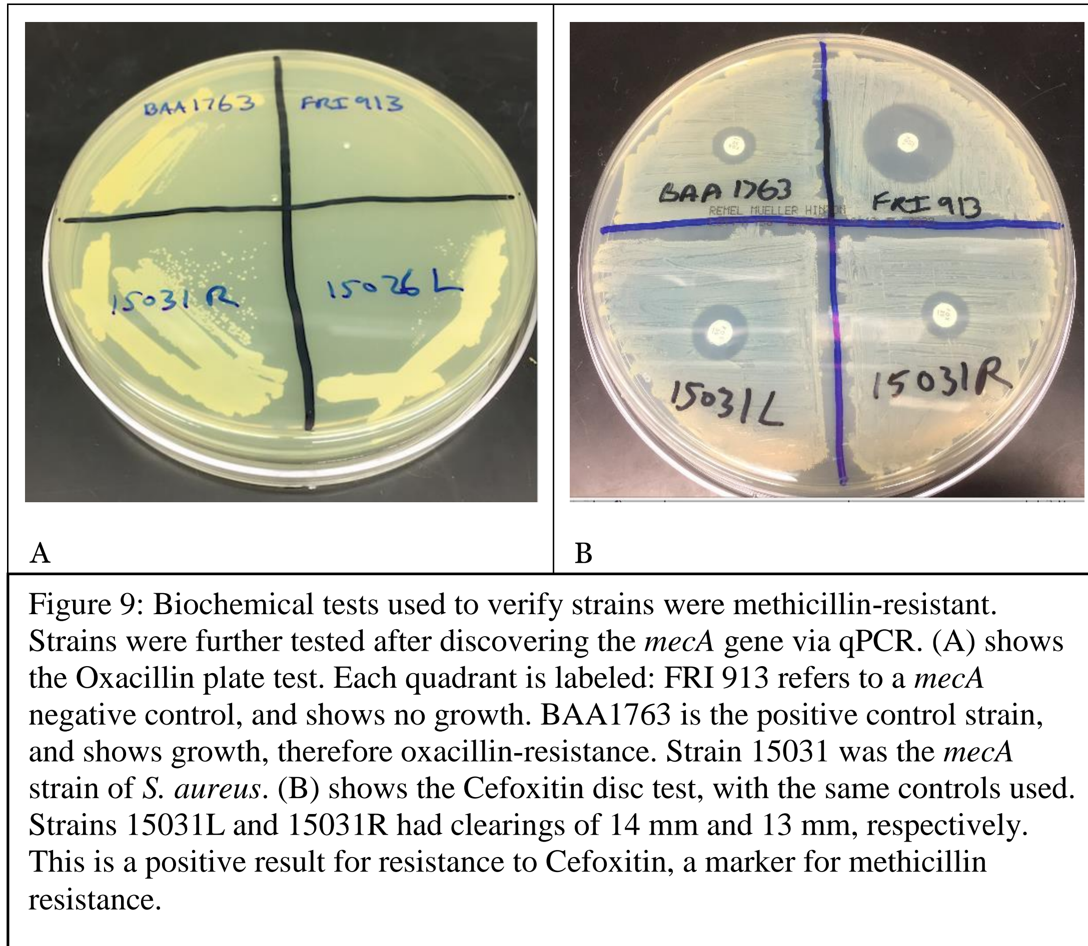


Figure 9: Biochemical tests used to verify strains were methicillin-resistant. Strains were further tested after discovering the *mecA* gene via qPCR. (A) shows the Oxacillin plate test. Each quadrant is labeled: FRI 913 refers to a *mecA* negative control, and shows no growth. BAA1763 is the positive control strain, and shows growth, therefore oxacillin-resistance. Strain 15031 was the *mecA* strain of *S. aureus*. (B) shows the Cefoxitin disc test, with the same controls used. Strains 15031L and 15031R had clearings of 14 mm and 13 mm, respectively. This is a positive result for resistance to Cefoxitin, a marker for methicillin resistance.

Each of the bacterial strains that required further testing was sent to the UPHS-Marquette laboratory streaked on agar plates. After analysis, the laboratory sent printouts of the results of antibiotic sensitivity testing that was performed, as well as the names of any identifiable bacteria, including the control strains (Figure 10). The control strains sent were BAA1763 and FRI913. Strain 15031 was determined to be methicillin-resistant (Figure 11).

Name		Specimen	BAA1763
Patient ID	NMU	Source	
Date of Birth		Ward of Iso	
Attd. Phys			

1 Methicillin Resistant Staphylococcus aureus

1 MRSA

Drug	MIC	Interps
Cefoxitin Screen	>4	POS
Ciprofloxacin	>2	R
Clindamycin	<=0.5	S
Daptomycin	<=0.5	S
Erythromycin	<=0.5	S
Gentamicin	<=4	S
Levofloxacin	>4	R
Linezolid	2	S
Moxifloxacin	>4	R
Nitrofurantoin	<=32	
Oxacillin	>2	R
Rifampin	<=1	S
Synercid	<=1	S
Tetracycline	<=4	S
Trimeth/Sulfa	>2/38	R
Vancomycin	2	S

A

Name		Specimen	FRI913
Patient ID	NMU	Source	
Date of Birth		Ward of Iso	
Attd. Phys			

1 Staphylococcus aureus

1 S. aureus

Drug	MIC	Interps
Amox/K Clav	<=4/2	S
Amp/Sulbactam	<=8/4	S
Ampicillin	<=2	N/R
Cefazolin	<=4	S
Cefoxitin Screen	<=4	NEG
Ciprofloxacin	<=1	S
Clindamycin	<=0.5	S
Daptomycin	<=0.5	S
Erythromycin	<=0.5	S
Gentamicin	<=4	S
Levofloxacin	<=1	S
Linezolid	2	S
Moxifloxacin	<=0.5	S
Nitrofurantoin	<=32	
Oxacillin	<=0.25	S
Penicillin	<=0.03	S
Rifampin	<=1	S
Synercid	<=1	S
Tetracycline	<=4	S
Trimeth/Sulfa	<=0.5/9.5	S
Vancomycin	1	S

B

Figure 10: Antibiotic sensitivity test results. (A) shows the positive control strain for *mecA* that was sent to UPHS-Marquette, BAA1763, with the result in line one showing that it is in fact Methicillin Resistant *Staphylococcus aureus*. (B) was the negative control for *mecA* that we supplied the lab, FRI913 (methicillin-sensitive *S. aureus*). The result in line one verifies that it is in fact methicillin sensitive *Staphylococcus aureus*.

Name		Specimen	15031R
Patient ID	NMU	Source	
Date of Birth		Ward of Iso	
Attd. Phys			

1 Methicillin Resistant Staphylococcus aureus

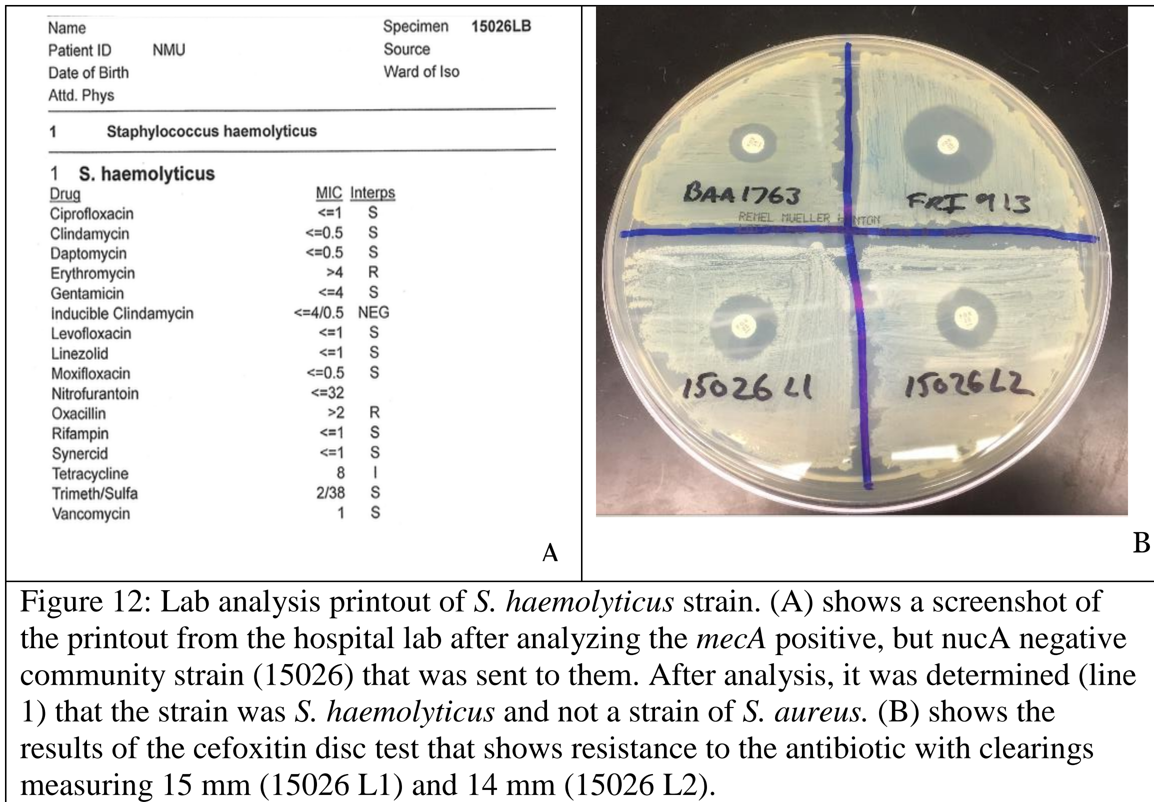
1 MRSA

Drug	MIC	Interps
Cefoxitin Screen	>4	POS
Ciprofloxacin	<=1	S
Clindamycin	<=0.5	S
Daptomycin	<=0.5	S
Erythromycin	<=0.5	S
Gentamicin	<=4	S
Levofloxacin	<=1	S
Linezolid	2	S
Moxifloxacin	<=0.5	S
Nitrofurantoin	<=32	
Oxacillin	>2	R
Rifampin	<=1	S
Synercid	<=1	S
Tetracycline	<=4	S
Trimeth/Sulfa	<=0.5/9.5	S
Vancomycin	1	S

Figure 11: Antibiotic sensitivity test results of the *mecA* community strain. After analysis, it was determined (line 1) that the strain was in fact methicillin-resistant *S. aureus*

Staphylococcus haemolyticus Strain

An unusual strain (labeled as "15026") was originally identified as *S. aureus,* as it was mannitol fermenting and beta-hemolytic, but it was not *nucA* positive, nor positive for Staphaurex. This strain also tested positive for *mecA*, so it was sent to the UPHS-Marquette hospital lab as well for further analysis using the same techniques previously described. The strain in question was determined to be *Staphyloccocus haemolyticus* (Figure 12). *S. haemolyticus* is a coagulase-negative staphylococci (CoNS) species that is closely related to *S. aureus*. *S. haemolyticus* is known for its high levels of resistance to many antibiotics, including methicillin. The same *mecA* gene is present on the staphylococcal cassette chromosome *mec* element (SCC*mec*) as the gene found in *S. aureus* [49].



Name
Patient ID NMU
Date of Birth
Attd. Phys

Specimen 15026LB
Source
Ward of Iso

1 Staphylococcus haemolyticus

1 **S. haemolyticus**

Drug	MIC	Interps
Ciprofloxacin	<=1	S
Clindamycin	<=0.5	S
Daptomycin	<=0.5	S
Erythromycin	>4	R
Gentamicin	<=4	S
Inducible Clindamycin	<=4/0.5	NEG
Levofloxacin	<=1	S
Linezolid	<=1	S
Moxifloxacin	<=0.5	S
Nitrofurantoin	<=32	
Oxacillin	>2	R
Rifampin	<=1	S
Synercid	<=1	S
Tetracycline	8	I
Trimeth/Sulfa	2/38	S
Vancomycin	1	S

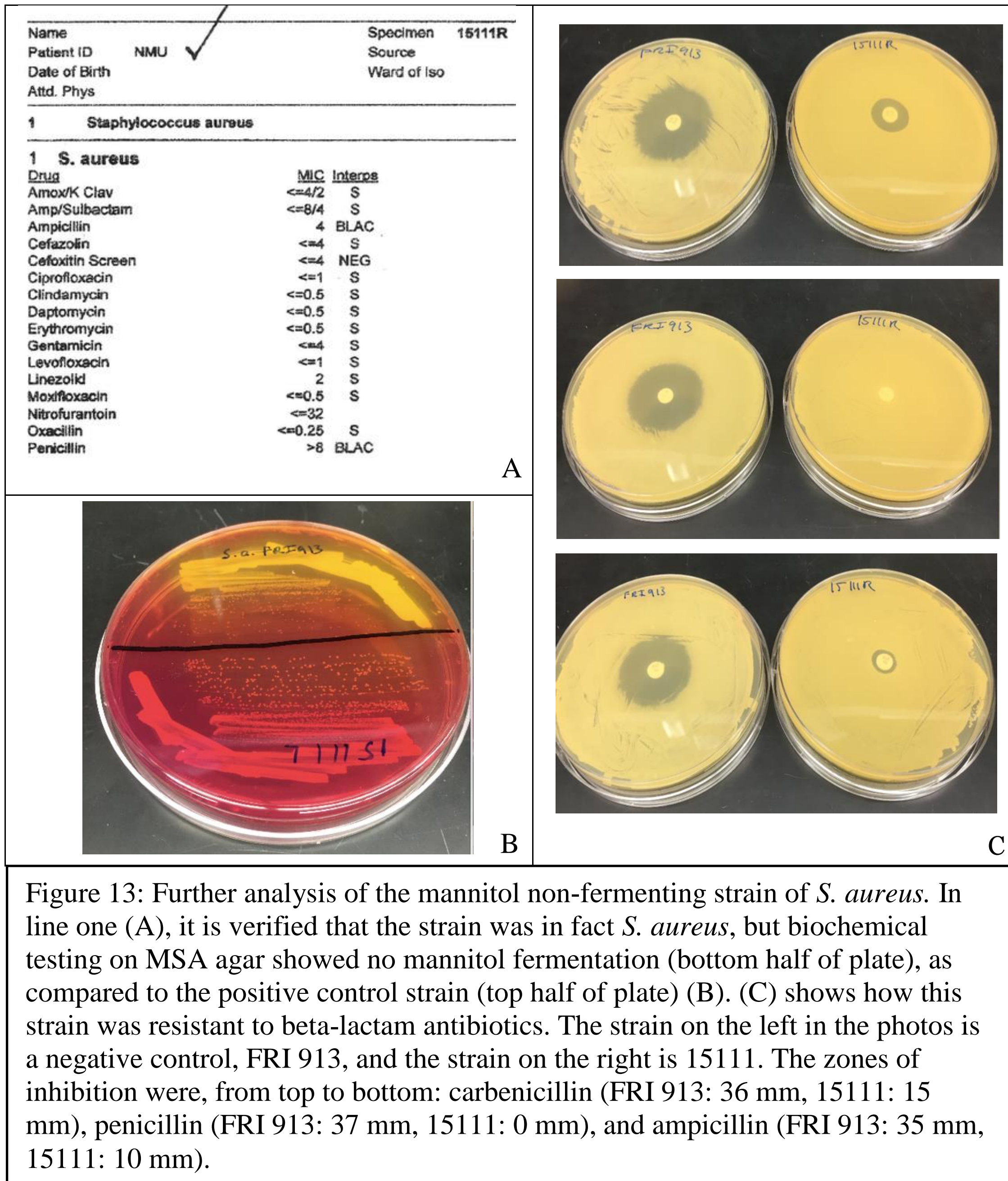
A

Figure 12: Lab analysis printout of *S. haemolyticus* strain. (A) shows a screenshot of the printout from the hospital lab after analyzing the *mecA* positive, but nucA negative community strain (15026) that was sent to them. After analysis, it was determined (line 1) that the strain was *S. haemolyticus* and not a strain of *S. aureus.* (B) shows the results of the cefoxitin disc test that shows resistance to the antibiotic with clearings measuring 15 mm (15026 L1) and 14 mm (15026 L2).

S. haemolyticus is associated with nosocomial infections, especially within the immunocompromised, including neonates [50], but it is not commonly found as a colonized strain within the nares of a healthy adult. This strain of *S. haemolyticus* was not included in the analysis in Figure 4 due to being a different species of *Staphylococcus* spp. While some of the toxin genes are similar in *S. aureus* and *S. haemolyticus*, there are definite genetic differences. Our qRT-PCR primers were species-specific by design to target for *S. aureus* specific genes, so this could have given us false-negative results when testing for genes that we know are usually present in *S. haemolyticus,* such as *nucA*.

Mannitol Non-Fermenting Strain

Another unusual strain was discovered within the collection samples, 15111. This strain presented as *S. aureus* because it had a typical *S. aureus* appearance on blood agar: round, yellow colonies that were beta-hemolytic. Strain 15111 also tested positive for both *nucA* and Staphaurex; however, it was mannitol non-fermenting – that it, although the strain grew on MSA agar, it did not produce the typical yellow pigment seen due to mannitol production. This strain was sent to the UPHS-Marquette laboratory for further testing, and was determined that it was a strain of *S. aureus* that was mannitol non-fermenting (Figure 13). According to Joe Levandoski, microbiology supervisor at UPHS-Marquette, less than 4% of *S. aureus* strains tested in our area are mannitol non-fermenting, which means that they do not contain the genes for mannitol fermentation.



Name		Specimen	15111R
Patient ID	NMU	Source	
Date of Birth		Ward of Iso	
Attd. Phys			

1 Staphylococcus aureus

1 S. aureus

Drug	MIC	Interps
Amox/K Clav	<=4/2	S
Amp/Sulbactam	<=8/4	S
Ampicillin	4	BLAC
Cefazolin	<=4	S
Cefoxitin Screen	<=4	NEG
Ciprofloxacin	<=1	S
Clindamycin	<=0.5	S
Daptomycin	<=0.5	S
Erythromycin	<=0.5	S
Gentamicin	<=4	S
Levofloxacin	<=1	S
Linezolid	2	S
Moxifloxacin	<=0.5	S
Nitrofurantoin	<=32	
Oxacillin	<=0.25	S
Penicillin	>8	BLAC

Figure 13: Further analysis of the mannitol non-fermenting strain of *S. aureus.* In line one (A), it is verified that the strain was in fact *S. aureus*, but biochemical testing on MSA agar showed no mannitol fermentation (bottom half of plate), as compared to the positive control strain (top half of plate) (B). (C) shows how this strain was resistant to beta-lactam antibiotics. The strain on the left in the photos is a negative control, FRI 913, and the strain on the right is 15111. The zones of inhibition were, from top to bottom: carbenicillin (FRI 913: 36 mm, 15111: 15 mm), penicillin (FRI 913: 37 mm, 15111: 0 mm), and ampicillin (FRI 913: 35 mm, 15111: 10 mm).

CHAPTER SIX: DISCUSSION & CONCLUSION

This study was the first to attempt utilizing qRT-PCR to identify which genes were present within community-colonizing strains of *S. aureus.* Five classes of Staphylococcal toxins (leukocidins, hemolysins, enterotoxins, toxic shock syndrome toxin, and exfoliative toxins) as well as methicillin-resistance were studied. Previous studies focused mainly on one or two of the classes at a time. Moreover, similar previous studies have focused primarily on hospital strains of the bacteria isolated after causing infection, or on strains found in contaminated foods. This study is important because it examined patterns of toxin carriage in isolates that have not previously been examined. Due to the nature of this study being a comprehensive report of all the different toxin types and their presence, several interesting combinations of toxin genes (or lack thereof) were discovered upon analysis.

A study by Mehrotra et al. (1999) looked at healthy human colonizing strains, and found that of 107 isolates tested, 24.3% had the *tst* gene [14]. This study showed similar findings, with 25.81% of the strains tested containing the gene. Interestingly, out of every strain of *S. aureus* that carried the *tst* gene, none of them also had the gene for any of the leukocidins. One study that detected the presence of toxic shock syndrome toxin along with the enterotoxins and exfoliative toxins found that *tst* presence is symptomatically related to enterotoxins, but there is no mention of its relationship with leukocidins [4]. Co-carriage of these two classes of toxins is not well-studied. It can be proposed that for a nasal colonizing strain of *S. aureus*, it might be disadvantageous to carry a toxin that works locally, like a leukocidin, as well as a systemic toxin, like *tst.* A strain carrying

both could potentially be extremely virulent and may cause in an aggressive infection, whereas it is more advantageous for nasal colonizing strains to be less virulent.

The study mentioned above found that 14% of the strains they tested contained only *tst*, 50% had *tst* in conjunction with *sea*, 11% had *tst* and *seb*, and 25% had *tst* and *sec* [4]. Comparatively, this thesis study found 13% of the strains contained only *tst*, and another 13% contained *tst* alongside *sea*. No strains presented with *tst* and any of the other four enterotoxins. These findings suggest that the presence of *tst* is not mutually exclusive with the presence of enterotoxins, and follows the same proposal suggested in previous studies that many healthy individuals can carry toxin-producing strains of *S. aureus* [14,51].

Within this study, there was a 100% prevalence for *hlgA* and *hlgC*. This is unsurprising as *hlgA*, *hlgB*, and *hlgC* are the most abundant extracellular proteins on *S. aureus* as identified in recent studies [52].

The *lukAB* gene was found in 54.8% of the strains isolated in this study, whereas *lukED* was only present in 41.9%. Interestingly, if a strain had the *lukED* gene, it co-presented with *lukAB* as well. However, *lukAB* was found without the presence of *lukED*. LukAB is one of the more prevalent leukocidins found in community strains of *S. aureus* [30], although the discovery of the toxin is so recent, there is much less data on *lukAB* carriage rates in *S. aureus*. Two strains (15037, 15071) co-carried both *lukAB* and *sea* (6%). One strain (15131) was positive for both *lukAB* and *seb* (3%), another strain (15126) was positive for both *lukAB* and *sec*, and a third strain (15154) was positive for both *lukAB* and *sed*. Unfortunately, a study comparing the prevalence of *lukAB* with other toxins has yet to be published due to the relatively recent discovery of *lukAB*. It can be

hypothesized that due to the high prevalence of LukAB in *S. aureus*, this leukocidin can be found co-carried with several other toxins. This is another example of why this study was so important in looking at the patterns in toxin carriage.

Panton-Valentine leucocidin was not present in any of the strains. This is not a particularly surprising finding, as Pvl is usually found in severe infections, so one would not expect nasal colonizing strains to be positive for Pvl. A study by Prevost et al. (1995) found only 2% of the strains tested contained Pvl [53,54]. However, the *S. aureus* strains tested in Prevost's study were isolated from aggressive skin infections, rather than the healthy nares of healthy members of a community.

The enterotoxins were found in a small percentage of strains, but this was expected as the strains were colonized from the nares, not from the gut. In addition, it was determined that no two enterotoxins were present together. With 32% of the strains positive for *sea,* only a handful of strains were positive for either *seb* (3%)*, sec* (6%)*,* or *sed* (3%)*,* and no strains contained *see.* The study by Mehrotra et al. (1999) found approximately 20% of the strains tested contained *sea*, 6% were positive for *seb*, 7% positive for *sec,* and 2% positive for *sed*; this study similarly found that no strains were positive for *see* [14]. These findings, that enterotoxin positive *S. aureus* colonizers are so commonly found in the nose, reinforce how important it is to follow food-safety regulations, such as wearing gloves and abiding by proper cooking/storage regulations.

These proteins function not only as gastrointestinal toxins, but as superantigens: they stimulate T-cell proliferation in the mammalian body [35]. As these two functions are located on two separate domains, they have different physiologic targets. Each of the enterotoxins are found in different locations on a prophage, and the prophages only carry

one type of enterotoxin [55]. In some instances, a plasmid can carry more than one type of enterotoxin at a time, but this is rarely observed. Additionally, most of the enterotoxins are found at a single locus in the *S. aureus* genome, making inheritance of more than one enterotoxin gene at a time very rare. Together, this may explain why we only found one type of enterotoxin, if any, in each strain of *S. aureus* isolated in this study.

Every strain out of the 31 collected was positive for both the *hla* and *hld* genes (100%), whereas only 27 of 31 strains were positive for *hlb* (87%). Comparatively, a study by Abdalrahman and Fakhr (2015) had similar findings: 97.7% of the strains isolated from chicken livers were positive for both *hla* and *hld*, and only 64.4% were positive for *hlb* [2]. These findings make sense because *hla* and *hld* are typically found together [56].

Zero of the isolated community strains were positive for *eta,* although this finding is unsurprising. Several studies have had negative results for exfoliative toxin when testing strains of *S. aureus* isolated from food or nasal passages [2,4,14]. Due to the severely infectious nature of strains that carry exfoliative toxins, one would not expect to find those virulent strains within the nares of an otherwise healthy host, similar to the expectations for Pvl.

Of the nasal isolates in this study, only one strain (15031) out of the 31 isolates was identified as methicillin-resistant (3.23%), aside from the one strain of methicillin-resistant *S. haemolyticus*. Literature shows that between 1.5-2% of the healthy, adult population is colonized for MRSA in the nares [57]. Out of the 141 total volunteers, this means there was MRSA colonization in only 0.7% of the population. Although slightly low compared to previously published literature, the sample size in this study was not

very large, so the percentages could be slightly higher. Further collections would need to be performed to gather a larger sample size.

While the work studying *S. pneumoniae* carriage was carried out in the CLS department and is not currently complete, it might be a good idea to consider the possibility of developing a vaccination against colonization by *S. aureus* if a correlation is found between *S. aureus* and *S. pneumoniae* carriage. In a study by Karauzum et al. (2013), they examined the possibility of developing attenuated LukS and LukF mutants as a vaccine possibility to defend against virulent *S. aureus* infection [58]. They reported successful development of two mutants that protected from lethal bacteremia caused by infecting mice with a CA-MRSA strain, USA300. Vaccination against Pvl seems to lead to decreased virulence in mice. Therefore, Pvl might be a useful vaccine candidate against extremely virulent strains of *S. aureus*. However, since Pvl is not carried by a high percentage of *S. aureus* strains, it would not likely function as a universal *S. aureus* vaccine.

A second study focused on developing a vaccine candidate based on the Hla toxin, which may be the better direction to go in due to the high prevalence of Hla (i.e. it was present in every strain of *S. aureus* in this study) [59]. The vaccine candidate was called AT-62aa, and was designed based on the heptameric crystal structure of Hla. It was tested in mice against pneumonia and bacteremia infection models using the Newman and USA300 strains of *S. aureus*, and showed high levels of protection in each model. A third study was focused on developing a vaccine specific to MRSA infection, but as Hla is a very important virulence factor in MRSA, this study focused on inducing high anti-Hla titers [60]. Results indicated that the vaccine inhibited lesion formation at the site of

infection. It was also successful at reducing the invasiveness of MRSA by preventing the spread of infection into other organs.

Interestingly, *hla* is also carried by some other pathogentic *Staphylococcus* species like *S. haemolyticus*. One study found that approximately 92% of *S. hameolyticus* isolates also carried *hla*[61]. There is about 2-5% difference in nucleic acid sequence between the *hla* gene carried by *S. aureus* and *S. haemolyticus*. It is currently unknown if a *S. aureus hla* vaccine would generate an antibody response that is cross-protective to *S. haemolyticus* infection. However, due to the high carriage rate of *hla* in *S. aureus*, *hla* seems very promising as a vaccine target. A general *S. aureus* vaccine could be useful in at-risk patients such as the elderly, the immunocompromised, or those with frequent skin diseases including infection by *S. aureus* or MRSA.

It is important to mention that more than one strain of *S. aureus* was isolated from some volunteers in this study, as we swabbed both nostrils. These strains were denoted by an 'R' or an 'L' to differentiate in which nostril the strain was discovered. Upon analysis, the same toxin gene was always found in each strain for the volunteer, which suggests that the strains were clonal. The *spa* gene (codes for the *S. aureus*-specific staphylococcal protein A) has a higher level of polymorphism, and is more useful in discerning whether or not two strains are clonal [2]. *S. aureus* isolates that have an identical or similar *spa* gene are more likely to be clonal or more closely related. To identify whether the strains in my study isolated from different nostrils are truly clonal, further *spa* typing would be an important next step. There are worldwide databases that have records of the *spa* types from various MRSA outbreaks and other *S. aureus* studies. It would be interesting to see

how related *S. aureus* isolates from the U.P. community are to those in other parts of the United States or world.

In the future, we hope to develop a stronger understanding of the virulence of *S. aureus* strains present in our community. The next plan for this project is to use next generation sequencing to sequence the genomes of several of the strains collected, and compare them to several *S. aureus* isolates from the hospital that are currently causing disease in the community. We could compare the toxin gene and the antibiotic resistance gene carriage between the two different groups of *S. aureus*. We could then study their genetic similarities and differences. This data could help develop an idea of how closely related the nasal colonizing strains are to disease-causing strains, and may give us key insights into *S. aureus* biology. Together, this data could help address the question regarding disease-causing probabilities of community strains.

To take it a step further, the genomic sequences of these strains can be compared to the national databases on *S. aureus* strains to hopefully gain a better understanding of how *S. aureus* spreads amongst humans. Overall, this study was one of the first to lay a roadmap toward understanding the difference between community strains of *S. aureus* and disease-causing strains, and as a result, can help to develop better options for not only the treatment, but the prevention of potentially fatal *S. aureus* infections.

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APPENDIX A

IRB Approval Letter

Memorandum

TO: Paul Mann, Cathy Bammert, Josh Sharp, Yuba Gautam
Clinical Lab Science, Biology

DATE: August 13, 2015

FROM: Brian Cherry, Ph.D.
Assistant Provost/IRB Administrator

SUBJECT: IRB Proposal HS15-678
IRB Approval Dates: 8/13/2015- 8/13/2016**
Proposed Project Dates: 8/1/2015-8/1/2016
"Relationship between methicillin-resistant *Staphylococcus aureus* nasal colonization and vaccination with a pneumococcal vaccine in adults"

The Institutional Review Board (IRB) has reviewed your proposal and has given it final approval. To maintain permission from the Federal government to use human subjects in research, certain reporting processes are required.

A. You must include the statement "Approved by IRB: Project #HS15-678" on all research materials you distribute, as well as on any correspondence concerning this project.

B. If a subject suffers an injury during research, or if there is an incident of non-compliance with IRB policies and procedures, you must take immediate action to assist the subject and notify the IRB chair (dereande@nmu.edu) and NMU's IRB administrator (bcherry@nmu.edu) within 48 hours. Additionally, you must complete an Unanticipated Problem or Adverse Event Form for Research Involving Human Subjects

C. Please remember that informed consent is a process beginning with a description of the project and insurance of participant understanding. Informed consent must continue throughout the project via a dialogue between the researcher and research participant.

D. If you find that modifications of methods or procedures are necessary, you must submit a Project Modification Form for Research Involving Human Subjects before collecting data.

E. **If you complete your project within 12 months from the date of your approval notification, you must submit a Project Completion Form for Research Involving Human Subjects. If you do not complete your project within 12 months from the date of your approval notification, you must submit a Project Renewal Form for Research Involving Human Subjects. You may apply for a one-year project renewal up to four times.

NOTE: Failure to submit a Project Completion Form or Project Renewal Form within 12 months from the date of your approval notification will result in a suspension of Human Subjects Research privileges for all investigators listed on the application until the form is submitted and approved.

All forms can be found at the NMU Grants and Research website:
http://www.nmu.edu/grantsandresearch/node/102

Amanda Wigand

Graduate Assistant, Grants and Contracts

Northern Michigan University

906-227-2437

APPENDIX B

Volunteer Informed Consent Statement

The Study
You are invited to be in a research study entitled, *The relationship between methicillin-resistant S. aureus nasal colonization and vaccination with a pneumococcal conjugate vaccine in adults*". The goal of this study is to explore whether vaccines that prevent pneumonia alter nasal flora of adults. This study is being conducted by Dr. Yuba Gautam, Dr. Paul Mann, Dr. Josh Sharp, Catherine Bammert, and graduate students Carol Kessel, Melissa Pierpont and Polly Hockberger from NMU. Up to 100 participants will be asked to be in this study.

Free to Choose
You are free to choose whether or not to be in this study. You may refuse to answer any questions you do not wish to answer. You may withdraw in part or in full from this study at any time without any ill will or penalty.

What to Expect
If you consent to participating in this research, you will be asked to complete a questionnaire which will take about 10 minutes of your time. You will also be asked to collect a swab from each of your nostrils. This process should take no more than 5 minutes.
Because we are an academic and not a certified or accredited, clinical laboratory, we are not legally allowed to share information with you regarding the bacteria we grow from your nasal swab.

Confidentiality
Your answers will remain anonymous. All raw data will be stored away from this consent form in a locked file drawer. Only the principle investigators listed above will have access to the data from this study. If the data is published, your name will not be linked to the data.
Additionally, the Northern Michigan University Institutional Review Board and federal reviewers may access our research data to review our research processes, if deemed necessary. In very rare cases, loss of confidentiality may occur if a court orders that research files or information be submitted as evidence in a legal matter.

Risks and Benefits
If you are uncomfortable collecting your nasal swab in an open area, we will make an accommodation to find a private area so that you will be more comfortable. While the risks associated with collecting a nasal swab are minimal, you may experience minor discomfort.

Your participation in this study will increase our knowledge regarding the relationship between vaccines that prevent pneumonia and the composition of nasal bacteria.

Questions about the Study
Please let us know now if you have any questions about this study. You may contact the researchers, as follows: Paul Mann (pmann@nmu.edu), Josh Sharp (jsharp@nmu.edu), Yuba Gautum (ygautam@nmu.edu), or Cathy Bammert (cabammer@nmu.edu).

Questions about Your Rights
If you have any questions about your rights as a research subject you may contact the NMU IRB Administrator, Dr. Brian Cherry, Assistant Provost of Graduate Studies and Research at Northern Michigan University 001-906-227-2300 or bcherry@nmu.edu.

I have read or the above informed consent has been read to me. The nature of risks, demands, and benefits of the project were made clear to me. I know that I may ask questions. I know I am free to withdraw from the project at any time without having any ill will or penalty. I also know that this informed consent sheet will be kept away from the project data to maintain confidentiality. Only the principle investigators or an authorized representative will have access to this consent statement. A copy of this consent statement will be given to me.

____________________ ____________________ __________________
Please print your name Please sign your name Date

I have taken care to explain the nature of the above project to the subject. I hereby confirm that to the best of my knowledge, the subject signing the informed consent form clearly understands the nature, risks, and demands of being a subject in this study. A language barrier, health problem, or other barriers have not precluded a clear understanding of his or her involvement in this project.

____________________________ __________________
Name Date

Thank you for your participation in this research study!

APPENDIX C

Volunteer Questionnaire

Q1. Age

- ❍ 18-30 years
- ❍ 31-40 years
- ❍ 41-49 years
- ❍ 50-59 years
- ❍ 60-69 years
- ❍ 75 years and above

Q2. Gender

- ❍ Male
- ❍ Female
- ❍ Other

Q3. Race/Ethnicity

- ❍ White
- ❍ Hispanic or Latino
- ❍ Black or African American
- ❍ Native American or American Indian
- ❍ Asian or Pacific Islander
- ❍ Other

Q4. Have you ever had frequent skin infections?

- ❍ Yes
- ❍ No
- ❍ Not Sure

Q5. Have you ever had frequent eye, nose, and throat infections?

- ❍ Yes
- ❍ No
- ❍ Not Sure

Q6. Have you ever been diagnosed with *S. aureus* or MRSA infection?

❍ Yes
❍ No
❍ Not Sure

Q7. Have you ever been diagnosed with *S. pneumoniae* infection?

❍ Yes
❍ No
❍ Not Sure

Q8. Do you have history of cancer, bone marrow, organ transplant, or immunosuppressive condition?

❍ Yes
❍ No
❍ Not Sure

Q9. Do you have a history of recent or frequent hospitalization?

❍ Yes
❍ No
❍ Not Sure

Q10. Have you ever been treated with antibiotics in the last 3 months? If yes, what kind of antibiotics?

❍ Yes
❍ No
❍ Not Sure

List antibiotics: ______________________

Q11. Have you been given the Pneumococcal vaccine? If so, do you know if it was the Prevnar vaccine?

- ❍ Yes
- ❍ No
- ❍ Not Sure
- ❍ Prevnar Yes__________, No ___________, Not Sure _________

Q12. I have read the inform consent. I am willing to participate in this study and give consent for a Nasal Swab.

Signature of the participant: ________________________________

APPENDIX D

Table of CT Values for Positive Signal of Toxins Obtained from This Study

Gene	Run	CT Values Observed
nucA	Collection One, 1st run	12.1, 12.7, 13.3, 12.9, 12.3, 12.8, 14.1, 13.2, 34.2, 31.1, 11.7, 11.0, 13.4, 11.8, 13.7, 13.2, 13.6, 13.6, 13.1, 11.8, 11.3
	Collection One, 2nd run	12.0, 12.6, 13.3, 13.0, 12.9, 12.8, 14.1, 13.3, 34.2, 31.2, 12.0, 11.6, 13.3, 12.0, 13.7, 13.4, 13.7, 13.7, 13.4, 11.4, 11.3
	Collection Two, 1st run	15.1, 16.3, 14.9, 14.9, 13.4, 15.0, 14.9, 13.5, 11.9, 11.2, 11.4, 11.6, 11.5
	Collection Two, 2nd run	15.2, 16.3, 14.9, 14.9, 13.4, 15.2, 15.1, 13.4, 12.0, 11.0, 11.4, 11.6, 11.5
	Collection Three, 1st run	16.0, 15.0, 17.1, 13.3, 15.5
	Collection Three, 2nd run	16.1, 15.3, 16.0, 13.4, 15.3
	Collection Four, 1st run	13.0, 13.1, 12.6, 11.6, 12.0, 12.2, 12.2, 12.5, 12.3, 13.1, 14.2, 12.1, 9.9, 11.0, 12.7, 12.6
	Collection Four, 2nd run	13.1, 13.2, 12.6, 11.8, 12.4, 11.9, 12.1, 12.3, 11.9, 13.1, 14.3, 12.2, 11.0, 10.8, 13.1, 12.4
mecA	Collection One, 1st run	35.4, 36.8, 0, 38.9, 0, 0, 0, 36.6, 13.4, 13.8, 12.9, 12.7. 34.6, 34.3, 0, 30.8, 0, 29.3, 34.5, 34.4, 30.8
	Collection One, 2nd run	36.1, 36.6, 0, 0, 0, 0, 0, 36.1, 13.5, 13.7, 13.0, 12.8, 35.1, 34.3, 0, 31.0, 0, 29.1, 34.3, 34.1, 31.1
	Collection Two, 1st run	0, 0, 0, 30.1, 34.3, 29.9, 29.9, 0, 31.0, 32.3, 0, 0, 0
	Collection Two, 2nd run	0, 0, 0, 30.6, 34.1, 30.2, 29.8, 0, 31.3, 32.7, 0, 0, 0
	Collection Three, 1st run	0, 0, 0, 0, 0
	Collection Three, 2nd run	0, 39.8, 0, 0, 0
	Collection Four, 1st run	36.6, 0, 0, 35.7, 0, 36.1, 36.7, 0, 0, 0, 37.1, 35.3, 36.7, 36.6, 38.5, 0
	Collection Four, 2nd run	36.4, 0, 0, 35.2, 0, 36.2, 36.7, 0, 0, 0, 37.3, 35.5, 36.8, 36.5, 38.2, 0

pvl	Collection One, 1st run	0, 0, 0, 43.7, 0, 40.7, 41.4, 0, 0, 0, 0, 0, 0, 0, 0, 0, 40.3, 39.8, 0, 42.6, 0
	Collection One, 2nd run	0, 0, 0, 0, 0, 42.2, 0, 0, 0, 0, 0, 0, 35.5, 0, 0, 36.1, 0, 0, 0, 0, 0
	Collection Two, 1st run	0, 0, 0, 0, 35.1, 0, 0, 0, 0, 42.1, 0, 40.6, 0
	Collection Two, 2nd run	0, 0, 0, 0, 35.4, 0, 0, 0, 0, 41.9, 0, 40.3, 0
	Collection Three, 1st run	0, 0, 0, 0, 0
	Collection Three, 2nd run	0, 0, 0, 0, 0
	Collection Four, 1st run	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 39.9, 0, 0, 0, 0
	Collection Four, 2nd run	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 41.1, 0, 0, 0, 0
lukED	Collection One, 1st run	31.4, 31.8, 34.8, 14.4, 42.7, 33.0, 0, 34.0, 34.9, 35.5, 36.1, 32.8, 34.8, 34.8, 33.2, 36.8, 43.6, 14.4, 14.0, 13.1, 14.5
	Collection One, 2nd run	31.2, 31.9, 35.4, 14.8, 34.7, 34.0, 0, 33.6, 37.8, 34.4, 33.3, 34.7, 43.4, 34.2, 33.6, 37.6, 34.8, 14.5, 13.6, 13.5, 13.3
	Collection Two, 1st run	15.1, 16.9, 35.1, 32.6, 32.5, 33.1, 34.9, 15.1, 12.7, 11.9, 12.4, 13.1, 12.6
	Collection Two, 2nd run	15.1, 17.0, 35.1, 33.0, 32.1, 33.0, 35.6, 15.1, 13.2, 12.9, 12.4, 13.1, 12.6
	Collection Three, 1st run	0, 40.1, 0, 0, 0
	Collection Three, 2nd run	0, 39.9, 0, 0, 0
	Collection Four, 1st run	33.1, 32.0, 14.1, 13.4, 13.9, 12.3, 14.1, 14.5, 13.0, 28.8, 31.1, 32.0, 31.9, 31.8, 14.4, 14.3
	Collection Four, 2nd run	33.2, 33.3, 14.3, 13.9, 14.1, 13.2, 14.4, 14.2, 13.6, 29.1, 30.0, 32.5, 32.0, 31.6, 14.3, 15.0
lukAB	Collection One, 1st run	30.7, 31.2, 30.7, 14.9, 13.8, 14.2, 31.3, 33.5, 34.4, 33.0, 32.4, 29.7, 14.5, 33.8, 32.9, 31.7, 34.8, 14.6, 14.7, 13.8, 14.5
	Collection One, 2nd run	31.1, 30.9, 31.2, 14.6, 13.2, 13.6, 30.7, 33.1, 35.2, 32.3, 32.7, 29.8, 14.5, 32.8, 33.1, 31.3, 35.3, 14.6, 15.6, 14.0, 14.6
	Collection Two, 1st run	14.9, 16.3, 36.3, 33.0, 33.8, 33.6, 33.6, 14.3, 12.7, 11.6, 13.4, 13.4, 12.7
	Collection Two, 2nd run	15.2, 16.1, 37.8, 33.4, 33.7, 33.5, 34.2, 14.0, 13.1, 11.4, 13.3, 13.1, 13.0

	Collection Three, 1st run	0, 0, 0, 0, 14.4
	Collection Three, 2nd run	0, 0, 0, 0, 14.0
	Collection Four, 1st run	0, 32.9, 13.3, 12.7, 13.3, 13.6, 13.6, 14.0, 14.0, 28.1, 32.8, 32.1, 13.2, 13.3, 14.5, 14.7
	Collection Four, 2nd run	0, 33.1, 13.5, 12.5, 13.0, 13.5, 13.6, 14.0, 14.1, 28.2. 31.8, 32.0, 13.0, 13.3, 14.5, 14.6
tst	Collection One, 1st run	31.1, 31.8, 31.1, 30.9, 32.0, 32.3, 15.0, 13.0, 0, 34.6, 31.3, 29.0, 30.1, 28.2, 13.8, 14.1, 14.7, 39.1, 37.3, 30.1, 30.8
	Collection One, 2nd run	29.5, 30.7, 30.9, 30.4, 31.4, 30.8, 15.5, 13.4, 0, 34.4, 30.5, 28.9, 29.9, 28.0, 13.9, 14.0, 14.8, 38.2, 35.4, 29.0, 29.1
	Collection Two, 1st run	30.9, 31.4, 14.1, 14.0, 31.8, 27.2, 30.1, 28.8, 30.1, 29.6, 31.0, 31.0, 40.7
	Collection Two, 2nd run	31.0, 31.2, 14.2, 14.4, 32.6, 27.4, 30.2, 29.2, 31.6, 29.2, 29.9, 31.1, 40.0
	Collection Three, 1st run	17.0, 15.3, 18.5, 14.7, 0
	Collection Three, 2nd run	16.5, 14.1, 18.1, 13.8, 0
	Collection Four, 1st run	14.5, 14.3, 30.1, 29.2, 31.3, 28.2, 31.1, 30.8, 29.7, 32.2, 29.1, 13.4, 33.5, 34.2, 29.1, 31.4
	Collection Four, 2nd run	14.1, 14.5, 30.2, 29.1, 31.1, 28.6, 31.1, 30.9, 29.5, 32.4, 29.3, 13.4, 34.0, 34.3, 30.0, 30.9
hla	Collection One, 1st run	16.2, 16.9, 17.5, 14.8, 13.9, 14.3, 16.9, 15.9, 36.7, 34.3, 15.4, 15.6, 15.4, 16.0, 16.2, 15.7, 16.2, 15.9, 14.2, 13.8, 13.9
	Collection One, 2nd run	15.9, 16.9, 16.8, 14.8, 24.0, 14.0, 16.7, 16.4, 35.1, 33.4, 15.1, 15.0, 14.9, 16.0, 15.6, 15.7, 15.8, 14.7, 15.0, 13.2, 13.9
	Collection Two, 1st run	16.1, 17.6, 16.4, 16.1, 17.2, 16.7, 16.3, 14.8, 13.8, 12.2, 12.9, 13.0, 13.6
	Collection Two, 2nd run	15.5, 17.2, 15.8, 15.9, 16.7, 16.0, 15.8, 14.7, 12.9, 12.2, 12.6, 13.1, 12.7
	Collection Three, 1st run	17.1, 15.6, 17.3, 14.0, 16.9
	Collection Three, 2nd run	16.8, 15.6, 17.2, 13.9, 15.8
	Collection Four, 1st run	17.1, 16.7, 14.6, 13.3, 13.6, 13.9, 14.1, 14.0, 15.5, 16.9, 17.1, 15.8, 12.5, 12.3, 14.4, 14.6
	Collection Four, 2nd run	17.3, 15.9, 15.1, 13.5, 13.5, 13.2, 15.1, 15.5, 16.1, 16.6, 16.9, 16.2, 12.4, 12.5, 14.4, 14.5

hlb	Collection One, 1st run	30.6, 31.5, 31.0, 14.3, 13.4, 13.5, 14.0, 13.7, 33.6, 33.4, 29.7, 27.4, 13.9, 27.2, 12.9, 13.3, 13.6, 14.4, 14.7, 13.3, 13.6
	Collection One, 2nd run	29.5, 29.4, 31.7, 13.9, 12.6, 12.8, 13.5, 14.4, 0, 42.8, 38.5, 35.4, 19.4, 31.0, 13.5, 13.4, 12.9, 14.2, 15.2, 12.6, 13.5
	Collection Two, 1st run	15.4, 16.3, 12.7, 13.3, 14.0, 13.5, 12.9, 13.9, 12.0, 11.0, 11.9, 11.9, 11.6
	Collection Two, 2nd run	15.1, 16.2, 13.2, 13.6, 14.1, 14.0, 12.7, 14.0, 12.3, 11.5, 12.0, 12.3, 11.4
	Collection Three, 1st run	17.4, 16.7, 16.3, 17.2, 0
	Collection Three, 2nd run	16.8, 15.6, 16.2, 17.0, 0
	Collection Four, 1st run	14.2, 13.4, 13.0, 12.1, 13.0, 13.3, 13.5, 14.0, 13.6, 13.9, 14.8, 12.8, 12.3, 12.2, 13.6, 13.7
	Collection Four, 2nd run	14.0, 13.5, 13.1, 12.0, 13.0, 13.5, 13.8, 13.9, 13.5, 14.1, 14.8, 12.7, 12.3, 12.3, 13.5, 13.8
hld	Collection One, 1st run	14.7, 15.2, 15.8, 15.7, 14.9, 15.3, 15.6, 13.9, 37.9, 33.6, 13.9, 13.9, 15.9, 14.3, 14.6, 14.3, 15.0, 16.2, 14.6, 14.6, 14.7
	Collection One, 2nd run	14.4, 15.3, 15.7, 15.6, 14.8, 14.9, 15.5, 15.0, 34.9, 32.1, 13.7, 13.8, 15.3, 14.1, 14.3, 14.5, 14.3, 15.2, 15.7, 14.1, 14.8
	Collection Two, 1st run	15.9, 17.7, 14.6, 14.4, 15.4, 15.3, 14.9, 15.3, 13.7, 13.3, 13.8, 14.0, 14.1
	Collection Two, 2nd run	15.7, 17.5, 14.3, 14.5, 15.6, 15.3, 14.9, 15.5, 13.8, 13.5, 13.7, 14.4, 13.6
	Collection Three, 1st run	15.2, 13.3, 16.1, 14.9, 15.0
	Collection Three, 2nd run	16.0, 14.0, 16.5, 15.3, 15.5
	Collection Four, 1st run	15.5, 14.2, 14.2, 13.9, 14.1, 14.0, 14.3, 15.1, 14.7, 15.6, 16.6, 14.7, 13.3, 13.4, 14.9, 15.0
	Collection Four, 2nd run	15.3, 15.0, 15.1, 13.9, 14.0, 14.2, 14.0, 14.3, 14.4, 15.2, 15.5, 14.7, 13.4, 13.6, 14.6, 14.8
sea	Collection One, 1st run	9.9, 10.3, 11.3, 30.2, 36.1, 33.9, 32.9, 30.7, 0, 0, 30.4, 27.5, 14.1, 27.2, 12.6, 12.9, 12.9, 26.9, 34.3, 0, 30.6
	Collection One, 2nd run	11.6, 11.1, 12.1, 30.5, 32.3, 32.8, 32.5, 29.4, 0, 31.1, 34.1, 32.3, 14.1, 35.2, 14.2, 13.3, 13.8, 27.9, 33.8, 34.3, 30.9
	Collection Two, 1st run	15.4, 17.9, 31.7, 28.8, 13.3, 12.6, 12.5, 25.4, 34.4, 27.7, 33.5, 33.2, 34.3

	Collection Two, 2nd run	15.5, 16.2, 31.5, 29.1, 14.6, 12.7, 12.6, 25.7, 32.4, 31.9, 32.4, 31.8, 32.4
	Collection Three, 1st run	16.1, 15.5, 17.3, 14.4, 0
	Collection Three, 2nd run	16.5, 14.9, 17.0, 15.2, 0
	Collection Four, 1st run	0, 0, 0, 37.7, 0, 0, 37.1, 35.0, 36.6, 14.3, 16.0, 15.5, 35.0, 37.2, 0, 35.5
	Collection Four, 2nd run	0, 0, 0, 36.0, 0, 36.5, 36.2, 36.1, 37.2, 15.3, 15.6, 15.7, 35.2, 36.0, 36.4, 35.1
seb	Collection One, 1st run	0, 41.1, 0, 0, 0, 0, 39.7, 43.4, 41.5, 42.3, 44.4, 0, 0, 0, 0, 42.7, 0, 0, 0, 43.9, 43.3
	Collection One, 2nd run	0, 40.6, 0, 0, 0, 0, 39.9, 40.7, 43.2, 42.1, 0, 0, 0, 0, 0, 43.6, 0, 0, 0, 42.9, 43.3
	Collection Two, 1st run	42.8, 0, 38.5, 40.7, 0, 41.4, 42.2, 44.3, 40.7, 41.3, 42.4, 41.0, 38.6
	Collection Two, 2nd run	43.1, 0, 0, 0, 0, 41.7, 42.1, 43.2, 36.2, 35.9, 35.0, 44.1, 34.0
	Collection Three, 1st run	0, 0, 0, 0, 15.1
	Collection Three, 2nd run	0, 0, 0, 0, 15.3
	Collection Four, 1st run	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
	Collection Four, 2nd run	37.9, 0, 0, 0, 0, 35.5, 37.2, 0, 0, 0, 0, 0, 0, 0, 0, 0
sec	Collection One, 1st run	38.2, 0, 0, 0, 0, 0, 0, 36.5, 0, 34.7, 13.7, 13.9, 0, 37.9, 0, 37.9, 37.5, 33.3, 37.1, 0, 42.9, 36.9, 37.0
	Collection One, 2nd run	39.4, 0, 0, 0, 0, 0, 0, 37.5, 0, 34.2, 34.1, 14.2, 14.6, 0, 38.5, 37.6, 38.6, 37.4, 41.6, 42.3, 37.2, 37.1
	Collection Two, 1st run	0, 0, 40.7, 44.2, 0, 0, 41.1, 41.4, 0, 36.9, 36.0, 37.7, 13.7
	Collection Two, 2nd run	0, 0, 41.0, 0, 0, 0, 41.0, 42.6, 0, 36.5, 36.4, 37.9, 13.5
	Collection Three, 1st run	0, 0, 0, 0, 0
	Collection Three, 2nd run	0, 0, 0, 0, 0
	Collection Four, 1st run	0, 0, 0, 0, 0, 0, 37.9, 40.1, 0, 0, 0, 0, 41.0, 0, 0, 38.7
	Collection Four, 2nd run	0, 0, 0, 0, 0, 0, 37.6, 40.3, 0, 0, 0, 0, 41.3. 0, 0, 38.4

sed	Collection One, 1st run	0, 0, 0, 0, 0, 41.0, 0, 44.0, 0, 35.6, 34.1, 0, 0, 0, 0, 0, 35.1, 0, 42.5, 0, 35.2
	Collection One, 2nd run	0, 0, 0, 0, 0, 41.4, 0, 44.8, 0, 36.1, 33.9, 0, 0, 0, 0, 0, 35.3, 0, 43.0, 0, 35.6
	Collection Two, 1st run	0, 0, 0, 0, 43.5, 0, 0, 0, 34.9, 32.6, 34.2, 35.3, 35.7
	Collection Two, 2nd run	0, 0, 0, 0, 0, 0, 0, 0, 35.0, 32.3, 34.4, 35.7, 36.0
	Collection Three, 1st run	0, 0, 0, 0, 0
	Collection Three, 2nd run	0, 0, 0, 0, 0
	Collection Four, 1st run	0, 0, 12.2, 33.5, 33.1, 0, 34.9, 0, 0, 33.6, 33.9, 33.8, 0, 0, 0, 33.3
	Collection Four, 2nd run	0, 0, 12.3, 33.4, 33.0, 0, 0, 0, 0, 34.1, 34.2, 33.9, 0, 0, 0, 33.2
see	Collection One, 1st run	42.5, 0, 0, 0, 0, 42.2, 0, 35.2, 0, 0, 0, 0, 0, 0, 0, 43.2, 0, 0, 41.7, 41.9, 0
	Collection One, 2nd run	33.7, 34.3, 35.2, 0, 0, 0, 0, 34.3, 35.2, 0, 34.2, 31.7, 0, 42.3, 35.2, 35.3, 41.2, 33.5
	Collection Two, 1st run	0, 0, 0, 41.1, 0, 0, 0, 0, 39.6, 38.8, 0, 0, 0
	Collection Two, 2nd run	0, 0, 0, 43.0, 0, 0, 0, 0, 38.9, 38.6, 0, 0, 0
	Collection Three, 1st run	0, 0, 0, 0, 0
	Collection Three, 2nd run	0, 0, 0, 0, 0
	Collection Four, 1st run	33.2, 0, 0, 0, 0, 0, 0, 0, 35.5, 0, 0, 0, 0, 0, 0, 0
	Collection Four, 2nd run	33.7, 0, 0, 0, 0, 0, 0, 0, 34.9, 0, 0, 0 0, 0, 34.8, 0
eta	Collection One, 1st run	42.6, 42.9, 0, 43.4, 42.8, 0, 0, 42.9, 43.3, 40.7, 41.2, 42.3, 0, 43.7, 42.8, 43.9, 0, 42.8, 0, 42.3, 42.1
	Collection One, 2nd run	42.0, 0, 42.3, 41.4, 40.7, 42.4, 0, 42.8, 42.2, 42.9, 39.5, 40.1, 43.1, 42.4, 0, 42.3, 44.3, 41.4, 43.3, 43.3, 41.1
	Collection Two, 1st run	0, 0, 41.7, 0, 0, 0, 0, 0, 40.7, 39.5, 0, 0, 40.5
	Collection Two, 2nd run	0, 0, 0, 0, 0, 0, 0, 0, 41.0, 39.1, 0, 0, 40.4
	Collection Three, 1st run	0, 0, 0, 0, 0

	Collection Three, 2nd run	0, 0, 0, 0, 0
	Collection Four, 1st run	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 39.1, 0, 0, 0, 0
	Collection Four, 2nd run	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 39.1, 0, 0, 0, 0
hlgA	Collection One, 1st run	13.4, 13.6, 14.4, 14.5, 13.5, 13.5, 13.8, 13.3, 32.5, 29.2, 13.1, 11.2, 14.6, 11.6, 13.1, 13.3, 13.8, 14.9, 14.5, 12.7, 13.0
	Collection One, 2nd run	13.3, 13.6, 14.1, 14.5, 13.6, 13.3, 14.0, 13.4, 36.1, 30.0, 13.2, 11.6, 14.6, 11.4, 13.0, 13.3, 13.7, 15.0, 14.6, 12.5, 13.0
	Collection Two, 1st run	15.1, 16.6, 12.5, 12.4, 13.8, 13.2, 13.1, 13.2, 11.8, 11.6, 11.5, 11.9, 12.0
	Collection Two, 2nd run	15.1, 16.3, 12.5, 13.2, 13.8, 13.2, 14.1, 14.9, 12.3, 11.2, 11.5, 12.2, 12.2
	Collection Three, 1st run	16.0, 16.5, 15.3, 17.4, 14.9
	Collection Three, 2nd run	16.1, 16.5, 15.8, 17.3, 14.0
	Collection Four, 1st run	12.2, 13.4, 14.9, 14.3, 15.2, 11.1, 11.9, 12.3, 13.4, 13.2, 14.1, 11.1, 11.0, 12.5, 14.3, 13.0
	Collection Four, 2nd run	12.2, 12.8, 14.9, 15.4, 15.3, 11.2, 12.5, 12.3, 12.8, 13.7, 13.4, 12.2, 11.9, 11.7, 13.3, 13.5
hlgC	Collection One, 1st run	13.5, 14.0, 14.2, 13.6, 13.6, 13.6, 14.3, 14.3, 33.4, 30.4, 13.5, 11.3, 15.3, 11.6, 13.5, 12.7, 13.1, 14.3, 14.6, 13.0, 13.0
	Collection One, 2nd run	13.3, 14.1, 14.2, 13.7, 14.1, 13.7, 14.2, 14.3, 34.0, 31.4, 13.4, 11.0, 15.3, 11.6, 13.3, 12.5, 13.1, 14.1, 14.7, 12.7, 13.2
	Collection Two, 1st run	13.7, 15.9, 12.0, 12.0, 11.3, 12.9, 13, 13.5, 11.6, 11.43, 11.6, 11.9, 11.5
	Collection Two, 2nd run	14.0, 16.2, 12.1, 12.3, 11.5, 13.2, 13.5, 13.6, 11.2, 11.5, 11.0, 12.6, 11.3
	Collection Three, 1st run	15.1, 16.4, 15.8, 17.6, 15.1
	Collection Three, 2nd run	16.3, 16.8, 15.3, 17.2, 15.5
	Collection Four, 1st run	14.1, 15.0, 15.3, 16.5, 16.2, 15.3, 13.2, 14.8, 14.2, 14.5, 13.8, 16.7, 15.1, 15.0, 14.1, 14.0
	Collection Four, 2nd run	15.3, 14.1, 16.8, 16.3, 16.5, 14.0, 14.2, 15.1, 14.0, 13.8, 13.6, 15.0, 15.9, 15.7, 14.3, 13.9