DEVELOPMENT OF METHODS USING CHROMAGAR™ MEDIA TO DETERMINE THE PREVALENCE OF STAPHYLOCOCCUS AUREUS AND MÉTHICILLIN-RESISTANT S. AUREUS (MRSA) IN HAWAIIAN MARINE RECREATIONAL WATERS.

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY MAY 2005

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CHAPTER ONE

Literature Review

A. **Staphylococcus aureus: An Overview**

1. **Basic characteristics of Staphylococcus aureus**

   *Staphylococcus aureus* is a gram-positive coccoid shaped bacterium (0.5 to 1.5 \( \mu \text{m} \) in diameter) that commonly occurs in grape-like clusters (Murray et al., 2002). This species is a non-motile, non-spore forming, facultative anaerobe that can grow in media containing 10% NaCl at temperatures ranging from 18°C to 40°C. It is catalase positive and can be distinguished from other members of the genus *Staphylococcus* by its ability to produce coagulase, a substrate that encourages blood plasma to clot. Members of this genus occur throughout nature and are primarily associated with skin, skin glands, and mucous membranes of mammals and birds. In particular, *S. aureus* is a common inhabitant of the anterior nares and at times the skin of humans.

2. **Staphylococcus aureus as a human pathogen**

   *Staphylococcus aureus* can cause a number of infections and remains an ominous pathogen due to its ability to cause overwhelming sepsis and death (Lowy, F. D., 1998; Murray et al., 2002). Most *S. aureus* infections involve cutaneous and subcutaneous tissues. Indeed, *S. aureus* is the most common cause of skin infections in the United States (Standiford, 2000; CDC, 2003). The prevailing *S. aureus* infections of the skin and other areas are listed in Table 1.1. The ability of *S. aureus* to cause such a number of disease symptoms is due to its many virulence factors (Table 1.2). Notably, this pathogen can secrete over 30 distinct extracellular proteins, many of which have been
characterized as toxic to humans. For instance, staphylococcal food poisoning is not an infection but rather a toxin mediated disease.

Transmission of *S. aureus* can occur through multiple routes including contact with infected persons, contact with asymptomatic carriers, air-borne spread, through contaminated objects, or consumption of contaminated foods. Cuts or abrasions of the skin provide a point of entry with subsequent local or generalized infection. The most important route for transmission appears to be through contact with open draining lesions. Such lesions disseminate organisms into their environment and then on to others. Another notable source of transmission occurs through contact with asymptomatic carriers who may have this pathogen in one or more body sites as well as their clothing. Carriage of *Staphylococcus aureus* occurs in approximately 25 to 30% of the population at a given time, providing a significant source for transmission in the community (Kluytmans et al., 1997).

3. *Staphylococcus aureus* infections and swimming in coastal waters

Staphylococci are hardy cells with thick peptidoglycan layers within their cell walls providing the ability to withstand many environmental stresses. These organisms are widespread in the environment and can be cultured from virtually all environmental surfaces (Melish, 1981). Environmental sources of *S. aureus* may serve as an important reservoir of contact diseases and some association has been reported. In one study, infections amongst athletes were implicated with *S. aureus* recovered from different surfaces in their athletic facility (Bowers and Domanski, 2004). Another study reported the survival of *S. aureus* on inanimate objects and subsequent transmission to patients in an intensive care unit in Hawaii (Bures et al., 2000). In addition, the inherent
The halotolerant nature of *S. aureus* makes it well suited for survival in marine environments. In contrast, the conventional enteric bacteria that are employed as indicators are not well adapted to survival in aquatic environments. Enteric bacteria, such as *Escherichia coli*, are sensitive to light, temperature, salinity, and low nutrient levels leading to low survival rates in marine waters (Solic and Krstulovic, 1994). Thus, *S. aureus* is adapted to multifarious conditions and survives significantly better in marine recreational waters than the current enteric indicators (Gabutti et al., 2000; Sotic and Krstulovic, 1994).

The elevated incidence of illness in swimmers vs. non-swimmers has been frequently reported (Cabelli et al., 1979; Chang and Pien, 1986; Efstratiou, 2001; Henrickson et al., 2001; and Seyfried et al., 1985). Gastrointestinal, respiratory and other diseases (skin, ear, eye, nose, and throat) are the most common forms of swimming related illnesses. Public health policies to limit these illnesses have been to monitor water for fecal indicator bacterial concentrations. These indicator bacteria are non-pathogenic and only indicate that the water is contaminated with sewage, which can be related to significant risks for illness. Acceptable levels of fecal indicators used as water quality standards have been established through epidemiological studies that relate incidence rates of illness to concentrations of fecal indicators in the water.

Being that gastrointestinal diseases are the most common swimmer related illnesses in the U.S., and that these diseases are usually transmitted via the fecal-oral route, fecal borne bacteria are used as the indicators for public health threats within publicly used water bodies. The most worrisome source of fecal contamination is sewage and such contamination is highly monitored. In the U.S., standards for acceptable levels of indicator bacteria in various types of water have been established by the
Environmental Protection Agency (EPA, 2002; EPA, 1986). Current water quality indicators include total coliforms, fecal coliforms, *Escherichia coli*, enterococci and fecal streptococci.

A notable amount of swimming related illnesses still occurs in waters seemingly void of fecal contamination, well within standard limits. This has led many to believe that bathers themselves can serve as the source of pathogenic bacteria. *Staphylococcus aureus*, being a common human inhabitant and well adapted to survival in aquatic environments, is a pathogen often mentioned in such discussions. A study conducted at popular marine beaches in Hong Kong (Cheung et al., 1991) reported a high correlation between bather numbers and *S. aureus* levels with minor correlation to conventional water quality indicators. In an epidemiological study, Seyfried et al. (1985) reported an apparent dose-response between concentrations of total staphylococci in recreational waters and illness amongst swimmers. Furthermore, many investigations have found *Staphylococcus aureus* to be a major bacterial contaminant of recreational waters with a high bather density (Cheung et al., 1991; Papadakis et al., 1997; Papapetropoulou and Rodopulou, 1994; Yoshpe-purer and Golderman, 1987). Many have recommended the use of total staphylococci in conjunction with enteric indicators for assessing recreational water quality. Unfortunately, the available methods to quantify concentrations of *S. aureus* in marine waters have been limited, impeding additional studies to correlate concentrations with subsequent illness.
B. Emerging Infectious Disease: Methicillin-Resistant *S. aureus* (MRSA)

1. History of antibiotic resistance in *Staphylococcus aureus*

   The treatment of *Staphylococcus aureus* infections is increasingly difficult, as they have become resistant to various antibiotics. The evolution of antibiotic resistance in *S. aureus* has been expeditious. Resistance to penicillin was first reported shortly after its discovery. It was not until the introduction of methicillin in 1960 that a reliable effective therapy against penicillin-resistant strains was available. Disappointingly, resistance to this semi-synthetic penicillin was reported the next year. In the United States, the first hospital outbreak of methicillin resistant *S. aureus* (MRSA) infections was reported in 1968 from Boston City Hospital (Barrett, 1968). An expansion of MRSA soon followed with an increasing number of methicillin-resistant *S. aureus* isolates recovered from clinical specimens in the 1980’s. Currently MRSA has a stronghold in hospitals to the extent that the majority of nosocomial isolates of *S. aureus* in the United States are methicillin resistant. Such isolates are currently referred to as hospital associated MRSA (HA-MRSA). Even more disconcerting are the recent reports of *S. aureus* with reduced susceptibility to Vancomycin, the last drug available in defense of multiple resistant *S. aureus* infections (Chang, 2003).

2. Community associated MRSA (CA-MRSA)

   Although most MRSA infections have been hospital associated, recently there have been increasing reports of community associated infections. When MRSA first appeared in the community, in most instances, either the affected patients had contact with healthcare facilities or individuals with hospital acquired MRSA (Mulligan et al., 1993). A change began taking place in the mid-1990’s in which the occurrence of MRSA
infections increased in otherwise healthy individuals with none of the previously known risk factors. These infections are commonly identified in children, and mostly the skin and skin structures are involved (IDSA, 2003). Outbreaks in such groups as Native Americans, prisoners, and competitive athletes have also been reported (CDC, 2003; CDC, 2003; Fey et al., 2003). Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) infections have been strikingly virulent, punctuated by the deaths of four previously healthy children (CDC, 1999). In the last decade CA-MRSA strains have hastened their rate of spread. They have now reached all regions of the U.S. and, in some regions, are the prevailing community pathogens. The emergence of MRSA within the community is a major threat with important clinical implications including complications with CA-MRSA due to treatment failure, difficulty in administration and expense of alternate treatments such as vancomycin, and the over-use of vancomycin, which will lead to increased antibiotic resistance in gram positive bacteria.

As with all *S. aureus* infections, asymptomatic carriers are important sources for transmission within the community. Unfortunately for MRSA there has not been any systematic surveillance to determine carriage in the general population. However, the rate of carriage amongst various groups has been described. A study conducted amongst children at several day care centers in Dallas found 3-24% were asymptomatic carriers of MRSA (Adcock et al., 1998). Whereas, Shopsin et al. (2000) reported only one in 500 (0.2%) outpatients from a New York hospital were positive for MRSA carriage, with the only positive patient having history of hospitalization and antibiotic use. Notably, these same authors also report familial transmission from the positive carrier. In addition, a review by Chambers (2001), it was stated that MRSA carriage rates of 25 to 50% have
been reported. The noticeable variability amongst these reports exposes the call for more information on MRSA in the community and the means by which further dissemination may occur.

3. Molecular biology of MRSA

Rather than producing the plasmid-mediated penicillinase that accounts for penicillin resistance, MRSA isolates acquired a gene integrated into the host chromosome which codes for a penicillin binding protein (PBP2’ or PBP2a). This protein has reduced affinity for binding β-lactam antibiotics while retaining its transpeptidase activity in peptidoglycan synthesis. The mecA gene, which encodes this protein, is carried on a large mobile genetic element called staphylococcal cassette chromosome mec (SCCmec). Mobilization of mecA occurs through the genes ccrA and ccrB, which are homologous to DNA recombinases of the invertase-resolvase family (Katayama et al., 2000). The protein products of these genes expedite lateral transmission of mecA by providing precise excision with site specific orientation upon integration. Lateral transmission mechanisms typically have reduced rates compared to the horizontally transferred events, such as plasmid encoded penicillinase, but the mobilization genes found in SCCmec may have contributed to a more hasty dissemination of MRSA into the community.

The transmission of methicillin resistance is thought to be a rare event, leading many to believe the emergence of MRSA in the community is due to escaped hospital strains rather than independent strains that acquired resistance through transfer of genetic materials (Bukharie, 2001; Chambers, 2001). However, molecular evidence suggests that HA-MRSA and CA-MRSA evolved through disparate events. So far, HA-MRSA isolates have exhibited four distinct types of SCCmec with the most common forms being
SCCmec I, II, and III. SCCmec I and II typically encode resistance to other antibiotics, accounting for the multi-drug resistant phenotype of most HA-MRSA. Whereas, CA-MRSA carry SCCmec type IV, a significantly smaller genetic element, which does not carry other antibiotic resistance genes. The appearance of SCCmec type IV in four distinct genomic backgrounds suggests ease of transfer for this cassette compared to types I, II, and III (Baba et al., 2002). In addition, Okuma et al. (2002) found a unique multi-locus sequence type in the internationally dominant CA-MRSA, which did not appear in any HA-MRSA tested. Furthermore, these authors suggest divergent evolution for resistance due to differing selection pressures between the highly competitive bacterial environments found in the community and antibiotic inundated hospital settings. Indeed, most CA-MRSA isolates maintain susceptibility to tetracycline, trimethoprom/sulfamethoxazole, and clindamycin, as well as lower levels of oxacillin resistance compared to hospital associated strains (Hindler, 2004; Okuma et al., 2002).

Although CA-MRSA are less likely to be multidrug resistant than hospital strains, they may be more virulent. A molecular study using sequencing data found 19 virulence genes in a CA-MRSA strain, which were not detected in the genomes of five HA-MRSA strains (Baba et al., 2002). The virulence factors these genes impart include a number of superantigens, such as staphylococcal enterotoxin H, and a leukocidin, Panton-Valentine leukocidin (PVL). Reports have indicated that PVL is detected in most SCCmec IV CA-MRSA isolates (Dufour, 2002; Gillett, 2002). Other investigators provide evidence that CA-MRSA have increased staphylococcal enterotoxin B and C production (Fey et al., 2003). Okuma et al. (2002) also found that the mean doubling time for the numerous CA-MRSA strains tested was significantly greater than that of the HA-MRSA strains.
These authors then suggested that the increased replicative fitness of CA-MRSA might impart greater infectivity for these strains. Clinically, CA-MRSA has also manifested its virulence as it has been implicated with many severe infections (CDC, 2003; CDC, 1997; Gosbell et al., 2001).

C. *Staphylococcus aureus* Infections in Hawaii

1. Clinical manifestations

In Hawaii, approximately 12,000 *S. aureus* isolates are recovered from diseased individuals each year (FenFang, 2004). Corresponding with national data, the most common infections involve the skin and/or soft tissue. In Hawaii, however, there is a high incidence of previous exposure to marine recreational waters prior to the onset of *S. aureus* infections.

Since 2000 the incidence of MRSA infections in Hawaii has risen to about 40% of all the *S. aureus* infections (Fenfang, 2004). Of greatest interest is the apparent increase in CA-MRSA. In 2000 around 20% of *S. aureus* isolates were MRSA recovered from outpatient settings which increased by 5% only two years later (Fenfang, 2004). In a retrospective study conducted with data from Hawaii acquired from 2001-2003, 28% of all MRSA infections studied were considered community acquired (Melish, 2004). Interestingly the same study indicated that Pacific Islanders were victims of 54% of these CA-MRSA infections, even though this group represents only 24% of the state's population. Again, most of these infections were of the skin and soft issues. A surveillance based study conducted at a military hospital in Hawaii found there to be 38% methicillin susceptible *S. aureus* (MSSA) carriage amongst asymptomatic outpatients with only 2% MRSA carriage (Kenner et al., 2000). However, a similar study that
included nearby military clinics found a 6% carriage of MRSA in patients before admission and 45% of *S. aureus* infections being MRSA (Fishbain et al., 2000). The incidence of MRSA infections in Hawaii has risen to disconcerting proportions, especially amongst community-associated disease. Clearly, there is a need for better understanding of the sources and mechanisms for transmission of this formidable pathogen.

2. Association with marine recreational waters

In Hawaii, infections associated with marine activity are commonplace and *S. aureus* is often implicated. Common infections of *S. aureus* include penetrating marine injuries, gastrointestinal illness, and pneumonia in near drowning victims (Chang and Pien, 1986). Recreational activity in marine waters is particularly common amongst Pacific Islanders, and includes surfing, swimming, fishing, and gathering seafood. An increased level of marine activity amongst this particular group may impart heightened incidence of infections if the waters they are exposed to serve as a source of transmission. First, in order to assess this assumption, it is important to determine if such waters are contaminated with the indicated pathogen. Indeed, previous studies of several popular marine beaches on Oahu determined that these beaches have high counts of *S. aureus* (Charoenca and Fujioka, 1993). Upon further evaluation, an association between bathing in such waters and subsequent infection with this pathogen was determined (Charoenca and Fujioka, 1995). In addition, Charoenca and Fujioka (1993) evaluated susceptibility of their *S. aureus* isolates and found that 1.8% were methicillin resistant. Further evaluations of these findings have also been hindered by the lack of feasible methodologies.
D. Available Media for Isolation of *Staphylococcus aureus*

1. Isolation of *Staphylococcus aureus* from clinical samples

The need for reliable recovery and detection of *S. aureus* from diagnostic specimens has been longstanding. This need has lead to the development of several media, including: phenylethanol, Baird-Parker, Enrichment medium, Chapman-stone, Columbia-CAN, Crisley-TPEY, Giolitti-Cantoni, KRANEP, Mannitol Salt, Lipovitellin Salt Mannitol, m-Staphylococcus broth, Staph 110 medium, Polymyxin Staphylococcus medium, Vogel-Johnson (syn. Tellurite Glycine Agar), Coagulase Mannitol Agar, Trypticase Soy Broth with Sodium Chloride and Sodium Pyruvate, and a new medium, CHROMagar Staph aureus.

1.1. CHROMagar™

CHROMagar™ Staph aureus (CSA) is a new chromogenic medium for the recovery and differentiation of *Staphylococcus aureus* (CHROMagar™, Paris, France). Several evaluations of this medium for use in clinical screening have used stock cultures of staphylococci and clinical specimens. Carricajo et al. (2001) reported the efficacy of screening specimens using CSA along with Staphychrom coagulase test compared to conventional methods that utilize rabbit plasma. In their evaluation they used 775 specimens (267 *S. aureus*: 263 detected on CSA). They reported that no false positives occurred using both CSA and Staphychrom and 15 false-positives with just CSA. Gaillot et al. (2000) also evaluated CSA and found CSA to have a better specificity and sensitivity than conventional methods. The sensitivity of this medium is a measure of its ability to differentiate *S. aureus*, while the specificity is its ability to differentiate non-*S. aureus*. Merlino et al. (2000) used
stock isolates of staphylococci to evaluate CSA. They reported 100% specificity and sensitivity for the *S. aureus* and coagulase-negative staphylococci isolates tested on CSA.

1.2. Media used for direct recovery of MRSA from clinical samples

Media for the recovery of MRSA have also been evaluated using clinical isolates. One such medium is Oxacillin Resistance Screen Agar (ORSA: Oxoid) which is comprised of peptones, 5.5% NaCl, lithium chloride, mannitol, aniline blue, and oxacillin at 2 µg/ml plus polymyxin B (50,000 IU/L). This chromogenic medium presumptively identifies MRSA isolates. Using clinical specimens, Apfalter et al. (2002) compared ORSA to phenyl-mannitol-salt-oxacillin medium (MS-Oxa), blood agar, and brain heart infusion (BHI). About a third of the MRSA recovered grew in BHI only and, overall, ORSA performed better than on mannitol salt agar with oxacillin. High numbers of false negatives, non-target colonies confirmed as MRSA, after 24 and 48 hours for both screening media were apparently due to very low bacterial cell numbers present. In another evaluation, Simor et al. (2001) compared ORSA to Oxacillin Mannitol-Salt agar (OMSA: MSA + 2µg/ml Oxacillin). They report ORSA as being slightly better at detecting MRSA with fewer false positives. A different report that compared ORSA to CHROMagar™ Staph aureus supplemented with 4µg/L of oxacillin (CSA+O) also found ORSA to perform better (Kluuytmans et al., 2002). This comparison resulted in fewer false positives on CSA+O after 24 hours from coagulase-negative staphylococcus (CNS) isolates (1.6%) than on ORSA (2.3%), but after 48 hours, this changed (8.9% false positive on CSA+O and 3.9% false positive on ORSA). False positives after both 24 and 48
hours with MSSA isolates on ORSA (0.8% false positive after 24 hours and 5.3% false positive after 48 hours) were more numerous than on CSA+O (0% false positive after 24 and 49 hours). Without supplementation, CSA was slightly better at detecting \textit{S. aureus} and had fewer false positives from CNS than ORSA. The authors suggested that the higher concentration of oxacillin in CSA+O inhibited some MRSA. Merlino et al. (2000) reported that CSA+O reliably detected multi-drug resistant MRSA strains. However, non-multi-drug resistant strains grew inconsistently and only four of the 12 isolates tested grew on CSA+O. They suggest that the intracellular co-transportation of methicillin with the chromogenic moiety may have contributed to nonspecific membrane disorganization or induced cell death.

2. Isolation of \textit{Staphylococcus aureus} from environmental water samples

Few reports have evaluated various media for the efficacy in recovering \textit{S. aureus} from water samples. Such media include: Baird-Parker, Enrichment medium, Chapman-Stone agar, Columbia-CAN agar, Crisley-TPEY agar, Giolitti-Cantoni agar, KRANEP agar, Mannitol Salt agar, Lipovitellin Salt Mannitol, m-Staphylococcus agar (with sodium azide), Staph 110 medium (with sodium azide), Polymyxin Staphylococcus medium, Vogel-Johnson, and BFR-0 agar (Alico and Dragonjac, 1986; Borrego et al., 1988; Klapes, 1983; Sato et al., 1995). These reports indicated BFR-0, Vogel-Johnson, and Baird Parker as good media for the recovery of staphylococci from water samples. Charoenca and Fujioka (1993) recovered \textit{S. aureus} from marine recreational waters using Vogel-Johnson agar supplemented with 0.005% sodium azide. At the time of this report, there had been no published description of an evaluation of media for the recovery of MRSA from water samples.
Table 1.1. Diseases commonly associated with *Staphylococcus aureus* infections.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Affected Organ System</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folliculitis</td>
<td>Skin</td>
<td>Base of follicle raised and reddened with small collection of pus beneath the epidermal surface.</td>
</tr>
<tr>
<td>Furuncles and Carbuncles</td>
<td>Skin</td>
<td>Deep cutaneous to subcutaneous inflammatory nodules.</td>
</tr>
<tr>
<td>Impetigo</td>
<td>Skin</td>
<td>Thickly crusted superficial infections.</td>
</tr>
<tr>
<td>Cellulitis</td>
<td>Skin</td>
<td>Acutely spreading, deep subcutaneous infection in which the infected area appears red, hot, and swollen.</td>
</tr>
<tr>
<td>Scalded Skin Syndrome</td>
<td>Skin</td>
<td>Abrupt onset of redness and inflammation around mouth, which covers body in 2 days (bullous impetigo is a localized form).</td>
</tr>
<tr>
<td>Staphylococcal food poisoning</td>
<td>Digestive</td>
<td>Consumption of enterotoxins causing abrupt onset of severe vomiting, watery diarrhea, and abdominal pain or nausea (~4 hours) that lasts less than 24 hours.</td>
</tr>
<tr>
<td>Toxic Shock Syndrome</td>
<td>Circulatory/Systemic</td>
<td>TSS toxin in blood, fever hypotension and diffuse rash, skin peels, impetigo on face and limbs, flattened red spot to pustule.</td>
</tr>
</tbody>
</table>
Table 1.2. The major virulence proteins produced by *Staphylococcus aureus*.

<table>
<thead>
<tr>
<th>Virulence Protein</th>
<th>Associated virulence mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teichoic acids</td>
<td>Species specific, phosphate-containing polymers that are covalently bound to the peptidoglycan layer and mediate attachment to mucosal cells by binding to fibronectin.</td>
</tr>
<tr>
<td>Protein A</td>
<td>Binds to Fc receptor of IgG1, 2, and 4, preventing antibody mediated immune clearance.</td>
</tr>
<tr>
<td>Clumping factor (bound coagulase)</td>
<td>Reacts with globulin plasma factor to form staphylothrombin, which catalyzes conversion of fibrinogen to fibrin.</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>Hydrolyzes hyaluronic acids, which are in connective tissue, facilitating spread.</td>
</tr>
<tr>
<td>Staphylokinase</td>
<td>Dissolves fibrin clots.</td>
</tr>
<tr>
<td>Lipases</td>
<td>Several different types are produced. Needed for cutaneous and subcutaneous infections as they hydrolyze lipids found in fatty tissue.</td>
</tr>
<tr>
<td>Exfoliative toxins</td>
<td>Includes staphylococcal scalded skin syndrome toxin (SSSS) and exfoliative toxins A and B (ETA and ETB). Both ETA and ETB are serine proteases, which split intercellular bridges in the stratum granulosum of the epidermis.</td>
</tr>
<tr>
<td>Enterotoxins</td>
<td>Includes enterotoxins A through J, excluding F (SEA to SEJ). Variants of SEC and SEG have been identified. SEA most commonly associated with disease, SEC and SED are the common forms found in milk, SEB causes pseudomembranous enterocolitis. All are stable to heating at 100°C for 30min and resist hydrolysis by gastric jejunal enzymes. Induces emesis and gastroenteritis (food poisoning). These are produced by 30-50% of all strains.</td>
</tr>
<tr>
<td>Cytolysins, hemolysin, and leukocidin</td>
<td>Includes α, β, and γ- hemolysins (hla, hlb, hlg) and Panton-Valentine leukocidin (PVL). These cause lysis of cells through pore formation. PVL has been associated with increased severity of skin infections and with necrotizing pneumonia (Dufour et al., 2002; Gillet et al., 2002).</td>
</tr>
</tbody>
</table>
A. An Introduction to the Problem

An association between water and disease has long been recognized. Recently, marine recreational water resources have encountered increased demands. Ingestion of such water has been associated with diseases mainly affecting the gastrointestinal tract. This is the most recognized mode of transmission for water borne diseases and the major source of contamination is feces, which can contain numerous enteric pathogens. Monitoring of swimming beaches for public health risks relies on microbiological standards that are based on concentrations of indicators of fecal pollution (*Escherichia coli*, coliforms and enterococci). However, a variety of non-enteric pathogenic microorganisms can be transmitted to humans through direct contact with natural marine recreational waters. These include human pathogenic bacteria carried into the water from the skin and upper orifices of swimmers and bathers, such as *Staphylococcus aureus*. Since this mode of transmission is not recognized as a major cause for disease, however, there has been little investigation into this problem and there are no approved methods or regulations for monitoring levels of these pathogens. This is unfortunate as in some areas, such as Hawaii, water borne diseases due to recreational use are more commonly a result of contact with non-enteric pathogens rather than ingestion of enteric pathogens. Several studies have suggested the use of *S. aureus* as an indicator in bathing beaches (see discussion in Chapter One, Part A, Section 3) as well as for swimming pools, as *S. aureus* are shed from humans (Greenburg, et al., 1992). In swimming pools,
staphylococci, streptococci, and *Pseudomonas aeruginosa* account for a large percentage of swimming pool associated illnesses and may endure the effects of chlorine (Seyfried et al., 1995).

**B. Identification of Problem**

In Hawaii there is a high incidence of *S. aureus* infections amongst recreational users of marine waters. In the first phase of this study an association was determined that infected persons have four times a greater chance of prior contact with marine waters than non-infected persons (Charoenca and Fujioka, 1995). In a related study, several beaches on Oahu were determined to have high concentrations of Staphylococci (>100CFU/100ml; Charoenca and Fujioka, 1993). However, the methods developed for those studies were too tedious and work intensive that it has not proven useful for routine monitoring by regulatory agencies. As a result, very little progress has been made with regard to further defining the role of *S. aureus* in marine recreational waters. The results of these studies show a need for a more feasible and reliable method to enumerate *S. aureus* from marine recreational waters.

More recently, infections caused by methicillin-resistant *S. aureus* (MRSA) have become a world wide problem. The development of MRSA is primarily blamed on the indiscriminate use of antibiotics. These pathogens are often resistant to multiple antibiotics, making treatment increasingly difficult and thus leading to increased incidence of morbidity and mortality. Historically the source of transmission has been nosocomial, but the epidemiology of MRSA is changing as community-acquired infections are significantly increasing (see discussion in Chapter One, Section B, and Section 2). All of the modes for the transmission of MRSA within communities are
unclear. However, if *S. aureus* can be transmitted to humans by using recreational waters that are highly contaminated with this pathogen, it is reasonable to assume that MRSA can also be transmitted by such means. This problem can be addressed by determining whether MRSA exists within the population of *S. aureus* in marine recreational waters. Thus, there is a need to develop a feasible method to enumerate *S. aureus* in marine recreational waters and to determine the prevalence of MRSA among these isolates.

**C. Identified Need and Hypothesis**

The problems with *S. aureus* and MRSA infections in humans and the possible transmission of these pathogens by recreational uses of water were identified in Part B. The identified need was for a feasible and reliable method to enumerate *S. aureus* and determine presence of MRSA from recreational waters to convey that community transmission of MRSA may occur through recreational uses of these waters.

The hypothesis is that if feasible methods are developed to enumerate these pathogens in marine recreational waters, then reliable concentrations of these pathogens at various beaches can be feasibly obtained. These kinds of data will fulfill the first requirement in assessing the risk of infection with these pathogens at various beach sites. Several sites should be assessed as different beaches have different factors that influence concentrations of these pathogens (e.g. bather density, amount of water circulation, nutrient input from land run-off, temperature variability, and solar radiation). Concentrations of pathogens in water represent basic information needed to develop water quality standards. These kinds of data are needed to convey acceptable risk levels of infection by these pathogens following the use of various beaches in Hawaii.
D. The Proposed Study

1. Goals

First, an evaluation and modification of the CHROMagar™ Staph aureus method was done in order to develop a feasible and reliable membrane filtration method to enumerate *S. aureus* and determine the prevalence of MRSA strains from marine recreational waters.

Second, *S. aureus* and MRSA isolates recovered from marine recreational waters were characterized and compared to clinical isolates in order to assess whether *S. aureus* isolates recovered from marine recreational waters are similar to those recovered from infected patients.

2. Objectives

To reach the goals of this study, the following objectives will be completed:

1. Determine the recovery efficiency for *S. aureus* from marine and buffered waters using the membrane filtration method to concentrate these bacteria on the membrane and CHROMagar™ Staph aureus as the specific medium to enumerate the concentrations directly based on distinctively colored colonies (mauve).

2. Determine whether additional agents can be used to enhance the selectivity of CSA for *S. aureus*.

3. Apply these methods to recover *S. aureus* from popular swimming beaches to determine the ranges of concentrations in recreational waters and to correlate their concentrations with the number of swimmers.
4. Develop methods to determine the prevalence of MRSA among the *S. aureus* isolated from natural marine recreational waters.

5. Characterize the *S. aureus* and MRSA isolates from marine recreational waters based on their antibiotic resistance profile, ribotyping, pulsed field gel electrophoresis, SCCmec typing and toxin genes analysis.

6. Compare the *S. aureus* and MRSA isolates from marine recreational waters with those recovered from clinical samples and determine if recreational waters are a likely source of transmission for *S. aureus* and MRSA.
CHAPTER THREE

Evaluation of CHROMagar™ Staph aureus for Use in Membrane Filtration Methods to Recover and Directly Enumerate Staphylococcus aureus from Coastal Waters

A. Objectives

The main objective for this part of the study was to evaluate CHROMagar™ Staph aureus (CSA) as the selective and differential medium with standard membrane filtration methods for the recovery and enumeration of S. aureus from marine recreational water samples. This evaluation included the addition of specific components to CSA for improving selectivity for S. aureus from marine water samples. The utility of adding each supplemental component to CSA media was tested by comparing the effectiveness of the modified medium to recover S. aureus from different marine water samples.

B. Materials and Methods

1. Sampling sites, collection of samples and use of membrane filtration method

For this study, the primary sampling sites selected were beaches previously determined to contain high concentrations of S. aureus by Charoenca (1991). Samples to evaluate CSA recovery and selectivity for S. aureus were collected at two marine recreational beaches (Kuhio and Sans Souci) and a single brackish water location (Ala Wai canal). Marine water samples were collected from the surface (approximately two feet deep) in sterile polyethylene containers (Nalgene) and immediately stored in an iced cooler. For all water samples, transportation to the laboratory and processing with standard membrane filtration methods (Greenberg, et al., 1992) occurred within six hours of collection. Appropriate dilutions of the samples, prepared in buffered water, were
filtered through 0.45 µm membrane filters (Gn-6 Gelman or MF-Millipore) using the membrane filtration technique. Followed by the placement of filters on test media (e.g. CSA), incubation for 24 - 48 hours at 37°C, and enumeration of resulting colonies. Selected colonies were further isolated for purification and confirmation (Part 3). Final concentrations were calculated using the mean value from countable plates (25-250 total colonies) and then multiplying this mean to adjust to corresponding CFU/100ml given the volume filtered.

2. Media used to recover *Staphylococcus aureus* from recreational waters

The lack of a direct method to enumerate this pathogen has hindered the regular monitoring of *S. aureus* in marine recreational waters (Klapes, 1983). The following commercially available medium was evaluated for the recovery efficiency and selectivity for *S. aureus* from marine recreational waters.

2.1. Selective medium: CHROMagar™ Staph aureus

CHROMagar™ Staph aureus (CSA: CHROMagar™ Microbiology; Paris, France) is a relatively new selective chromogenic medium that allows direct detection of *S. aureus* by colony color (mauve) after 24 hours of incubation. This medium was determined to be reliable for the efficient recovery of *S. aureus* from clinical specimens. Many clinical laboratories currently use this medium. Peptones (40 g/l) in the medium promote bacterial growth, while its salt content (25 g/l) and a particular ingredient within the chromogenic mix (2.5 g/l) are selective for *S. aureus*. The recipe for the chromogenic mix is proprietary, but contains four chromogenic agents (phosphate, glucoside, galactoside, and glucuronide) and an iron-chelating agent (Deferoxamine; Rambach, 2000). Chromogenic agents are compounds that
change color under the effect of the enzymatic system of a specific strain of bacteria. With this medium, the *S. aureus* colonies turn mauve due to an enzyme system that acts upon the chromogenic phosphate compound. In a similar manner, the other chromogenic compounds infer various other colors to non-target colonies. The addition of deferoxamine, according to the inventor, inhibits *S. epidermidis*, making the medium able to distinguish this species from *S. aureus*.

2.1. Non-selective medium: tryptic soy agar

Tryptic soy agar (TSA) is a non-selective medium that contains excess nutrients and minimal salt. This medium is capable of growing a number of bacteria and readily grows *S. aureus*. On TSA, colonies of *S. aureus* appear as circular, entire, smooth, convex, semi-transparent, cream or golden colored colonies with a diameter of one to three millimeters. This morphology does not adequately differentiate *S. aureus* from other bacterial cultures but a golden color is indicative of this species. Due to the high nutrient content of TSA, nearly all viable bacteria capable of growth on this medium presumably form colonies. Thus, maximum recovery of *S. aureus* is expected using this medium.

3. Biochemical tests used to confirm presumptive target colony as *S. aureus*

CHROMagar Staph aureus™ recovers a target colony (mauve), presumptively identified as *S. aureus*. Evaluations using clinical samples found this medium to be highly sensitive and specific for the identification of *S. aureus* as mauve colored colonies. However, there have been no evaluations using environmental samples, and the diversity of bacteria found in such samples is greater than that found in clinical samples. Therefore, several of the mauve colonies recovered on CSA from environmental samples
must be further tested with biochemical tests to confirm their identification. Figure 3.1 illustrates the steps for the characterization and identification of *S. aureus*. First characterization of bacterial isolates was gram stain and the catalase test using 3% hydrogen peroxide. Gram-positive cocci that gave a positive catalase reaction were further examined by a *S. aureus* specific latex agglutination test (BBL™ Staphyloslide™) and coagulase test.

Latex agglutination is a reliable test procedure for the rapid differentiation of *S. aureus* from coagulase-negative staphylococci (Baker et al., 1985; Essers and Radebold, 1980). This test is used for the detection of coagulase (clumping factor) and protein A from primary cultures suspected to be *S. aureus*. The Staphyloslide latex test kit used in this study was performed by mixing 4-5 colonies from a suspected *S. aureus* culture with one drop of both test and control reagents on the test card. Then, rotating the card by hand and determining if agglutination with clumping factor and protein A on blue latex particles formed (test positive). *Staphylococcus aureus* colonies, when mixed with blue latex reagent, agglutinated within 20 seconds into visible clumps.

Coagulase is a substance that activates the clotting of plasma, and nearly always produced by *S. aureus* strains. There are two types of coagulase: 1) free coagulase is an extracellular enzyme, 2) bound coagulase (clumping factor) remains attached to the cell wall of the bacterium. The coagulase slide test detects only bound coagulase. The slide test done by mixing 4-5 colonies from a bacterial culture with one drop of rabbit plasma with EDTA (Becton Dickinson) on a slide. After rotating the slide by hand, white clumps of clotted plasma indicated the presence of free coagulase. The coagulase tube test detects both bound and free coagulase. This test is the most frequently used method
for detecting the coagulase enzyme because of its accuracy and is the most widely used test that differentiates coagulase-negative non-pathogenic species from coagulase-positive pathogenic *S. aureus*. The tube test was performed by inoculating 0.5 ml of the rehydrated plasma with 4-5 colonies from an overnight bacterial culture. Gently mixed the tube and incubated in a water bath at 35°C. Formation of a clot was examined after 4 hours. If a clot was not visible, incubation continued until 18-24 hours. The formation of a clot can be difficult to visualize and various degrees of clot formation were recorded. As recommended by the manufacturer, used a marking system of 1+ to 4+ to indicate the degree of clotting. Considered small unorganized clots as 1+ clot and this number designation increases with increasing size and organization of the clot up to a 4+ clot, which remains stable in the bottom of the test tube when inverted. One report recommends the use of only 4+ reactions as indicative of *S. aureus* (DiSalvo, J.W., 1958). In this study only isolates that formed clots >1+ were considered as positive.

4. Determining recovery efficiency of media for *S. aureus* from marine waters

Several investigators have evaluated different media for their ability to recover *S. aureus* from water (Alico and Dragonjac, 1986; Alonso et al., 1999; Borrego, et al., 1987; Borrego, et al., 1988; Hoi et al., 1998; Stengren and Starzyk, 1984). In these evaluations, the efficiency of recovery was determined by percent recovery. This was done by preparing suspensions of pure cultures and/or using water samples, filtering appropriate volumes of these suspensions or samples using membrane filtration techniques, and subsequently plating onto test media and a reference medium. Then, comparing average colony counts on the test media to the reference medium. Similar procedures were employed in this study where percent recovery was calculated from the ratio of colony
counts on selective media as compared to non-selective medium (100% recovery). In order to determine recovery efficiency of a particular selective media for *S. aureus*, test samples were prepared by adding *S. aureus* cells to sterile buffered water and filter sterilized seawater. Appropriate amounts of these seeded waters were then concentrated onto 0.45 μm membrane filters (Gelman GN-6). These filters were then placed onto selective and non-selective media, incubated, and enumerated resulting colony forming units.

A similar procedure was also performed using raw seawater. In this case, an additional sample of sterile water was seeded with the same concentration of cells. Using the same membrane filtration techniques non-selective media were inoculated with this sample to compare with concentrations of *S. aureus* recovered on the selective media from seeded raw seawater. In addition, unseeded raw seawater was filtered and the membrane plated onto selective media to approximate the amount of existing *S. aureus* in the seawater. Then, target (mauve colonies, presumptively *S. aureus*) and total colony counts recorded from selective media plates inoculated with unseeded and seeded raw seawater. These counts were used to determine the selectivity (the degree to which a medium effectively recovers a target organism while inhibiting the growth of non-target, non-*S. aureus*, organisms) for the particular selective media. Selectivity was determined by the percentage of inhibition for non-target colonies (not typical mauve morphology of presumptively *S. aureus* colonies) on the selective media as compared to concentrations of non-target colonies (not *S. aureus*) recovered on non-selective media (100%). In order to estimate the concentrations of non-target cells on the non-selective media, the amounts of *S. aureus* cells from seeded sterile water on non-selective media and unseeded raw
seawater on the selective/differential test media were subtracted from the total bacterial counts on the non-selective media plated with seeded seawater. These approximated concentrations were then compared with concentrations of non-target colonies recovered on the selective/differential media in order to determine the degree to which it inhibits non-target cells.

5. Special reagents and strains of S. aureus used in evaluation of selective medium

Certain compounds have been found to enhance the growth of S. aureus and others to inhibit non-staphylococci bacteria (Table 3.1). In order to determine if such compounds may be useful in enhancing the recovery and selectivity of certain S. aureus specific media, a range of concentrations of selective and enhancing agents were added and the modified media subjected to the same recovery and selectivity tests as described for the unmodified media. In such cases, recovery was also compared to unmodified CSA (100%).

Several different strains of S. aureus were used when preparing samples to assay different media. This was done in order to assess if inherent differences within a S. aureus population will affect the selectivity or recoverability of the media tested (Table 3.2).

Filter-sterilized seawater was prepared by using a sterile vacuum flask to collect seawater passed through a 0.22 μm membrane filter (Membra-Fil by Nucleopore). Once sterilized, this seawater was used immediately in seeding experiments or refrigerated in a sealed sterile container until use. Buffered water (pH 7.2 ±0.5) was prepared by heat sterilizing a measured amount of distilled water and adding magnesium chloride (0.04055%) and potassium dihydrogen phosphate (0.00425%).
Table 3.1. Agents reported to improve the selective recovery of *Staphylococcus aureus* from environmental samples.

<table>
<thead>
<tr>
<th>Target Organism</th>
<th>Selective Agents</th>
<th>Enhancement of Growth</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>Sodium Azide, Polymyxin B, Potassium-Thiocyanate</td>
<td>Glycine, Sodium Pyruvate</td>
<td>Borrego et al, 1987; Chaorenca and Fujioka, 1993</td>
</tr>
</tbody>
</table>

Table 3.2. Characteristics of *Staphylococcus aureus* strains used to seed seawater in various tests media tests.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Source</th>
<th>Type</th>
<th>Media Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25923</td>
<td>Standard stock</td>
<td>S</td>
<td>CSA, CSA with azide (all concentrations), CSA with both azide and oxacillin at various combinations of concentrations, CSA with azide (a few concentrations) and glycine (includes CSA+A+G)</td>
</tr>
<tr>
<td>6/1</td>
<td>Clinical</td>
<td>R</td>
<td>CSA, CSA with azide (most concentration; includes CSA+A), CSA with both azide and oxacillin at various combinations of concentrations, and CSA with different concentrations of sodium pyruvate or potassium thiocyanate</td>
</tr>
<tr>
<td>6/4</td>
<td>Clinical</td>
<td>R</td>
<td>Same media as for 6/1, except CSA with sodium pyruvate or potassium thiocyanate</td>
</tr>
<tr>
<td>10-1</td>
<td>Environment</td>
<td>R</td>
<td>Same media as for 6/4</td>
</tr>
<tr>
<td>17-76</td>
<td>Environment</td>
<td>R</td>
<td>CSA, CSA+A, and CSA+P; as well as all these with glycine</td>
</tr>
<tr>
<td>28-37</td>
<td>Environment</td>
<td>R</td>
<td>CSA, TSA with NaCl</td>
</tr>
</tbody>
</table>

Type = oxacillin susceptible (S) or oxacillin resistant (R); Environment = recovered from seawater sample (ID for these corresponds to Experiment # - Isolate # in Appendix D); Clinical = recovered from patient specimens and identified at commercial laboratory; CSA = CHROMagar™ Staph aureus, CSA+A = CSA with 0.0035% sodium azide, CSA+A+G = CSA+A with 12 g/L glycine, CSA+P = CSA with polymyxin B, TSA = tryptic soy agar.
Figure 3.1. Flow chart for confirmation of *Staphylococcus aureus* (TSA = tryptic soy agar, + = positive, +/- = weakly positive).
C. Results and Discussion

1. Recovery efficiency of CHROMagar™ Staph aureus (CSA) for S. aureus from seeded sterilized waters using membrane filtration methods (Phase 1)

The first step in the evaluation of the medium was to determine the efficiency of the method to recover S. aureus under the ideal conditions of seeding S. aureus into sterilized water samples. Various S. aureus strains were seeded into 10 buffered water samples and 12 filter-sterilized seawater samples. Using membrane filtration techniques, portions of these samples were plated onto CSA and TSA in duplicate and sometimes triplicate, and then incubated as previously described. Colonies of S. aureus on CSA appeared as small (1-2 mm) to medium (2-3 mm), opaque, mauve (pinkish purple) colored colonies with a diffuse mauve colored halo (Figure 3.2 and Figure 3.3). On TSA, these colonies appeared as small to medium, semi-opaque, cream to golden colored colonies.

The mean percentage of S. aureus colonies recovered on CSA compared to TSA was lower from buffered water than from filter sterilized seawater, 78.3% (95% C.I. = 70.0 to 92.2%) and 98.6% (95% C.I. = 89.7 to 111%) respectively (Table 3.3). However, the range of percentages included in 95% confidence intervals overlaps between these artificially seeded water samples. Thus, the recoverability for S. aureus on CSA from these waters is not significantly different but some discussion for the lower recovery from buffered water is warranted.

Buffered water is often suggested as the diluent when performing microbiological analyses of water samples (Greenberg et al., 1992). This solution was used for seeding experiments because it is a prepared solution, limiting variability, and is recommended in
standard methods for dilution. Differences in seed preparation may be one explanation for the differences in \textit{S. aureus} recovery from these two types of waters. Inherent variability in seed preparation may have resulted in different stresses on cells and thus recoverability. None of the seeding experiments used both types of water with the same seed preparation, so no direct comparison is available. Therefore, variations in stress level amongst prepared batches of cells cannot be discounted as a cause for different recovery percentages from these two waters. Another possible explanation is that the filter-sterilized seawater, although itself highly limited in nutrients, most likely contains more nutrients than the lab prepared buffered water; therefore, may have sustained healthier cells leading to their improved recovery on CSA.

Overall, both the percentages of recovery (78.3\% and 98.6\%) are high. For comparison, Borrego et al. (1987) reported that their medium, BFR-O, recovered over 75\% of staphylococci from phosphate buffered saline and that this was an acceptable level of recovery. Therefore, under Phase one conditions, CSA effectively recovered \textit{S. aureus} cells from water samples using membrane filtration methods.

2. Recovery efficiency of CSA for \textit{S. aureus} from seeded natural waters using membrane filtration methods (Phase 2)

Natural waters contain many different microorganisms. Ocean waters have been reported to contain more than one-hundred thousand bacteria cells per milliliter (Whitman, et al., 1998) and many of these are novel organisms that have yet to be cultured (Rappe and Giovanni, 2003). Given this high concentration of cells and intense variety of bacteria present in natural seawater, it was important to determine if recovery efficiency of CSA for \textit{S. aureus} was still adequate when recovered from natural seawater.
A total of ten raw marine water samples, nine coastal seawater samples and one brackish canal water sample, were seeded with various strains of *S. aureus* and then analyzed in the same manner as for seeded sterile waters (see Phase one). For these samples, the counts for *S. aureus* on TSA were estimated and not directly counted as in Phase one (see Materials and Methods section, Part one for details). As a result, it was estimated that 44.7% (95% C.I. = 33.1 to 82.9%) of the *S. aureus* cells were recovered on CSA compared to TSA (Table 3.3). In addition, the recovery efficiency is much lower from natural water (44.7%) than the sterilized waters (78.3% and 98.6%; Table 3.3). This is likely caused by competition and predation, which would be highly limited in the seeded sterilized water samples. Also apparent in these data is the increase in recovery variability in natural waters compared to filter-sterilized waters, evidenced by greater range of confidence intervals. Variability is to be expected in natural waters where parameters such as nutrient content, presence of toxic compounds, and prevalence of competitors and predators will vary amongst different samples. It should also be mentioned that some variability can be attributed to the method used in determining the recovery, as it is not a direct count of *S. aureus* on TSA but an estimate determined from seeding concentrations (counts on TSA from buffered water) and existing concentrations (counts on CSA from unseeded seawater). The difference in *S. aureus* recovery between natural and sterilized seawater is significant, as the range included in the confidence intervals between the two do not overlap. Therefore, the presence of other organisms and the difference in determining recovery are important factors to consider. On the other hand, the confidence intervals for buffered water and natural seawater do overlap, indicating that variability may also occur due to seed preparation. Although the
efficiency of *S. aureus* recovery on CSA from natural seawaters appears to be highly variable, this may be attributed in part by the methods employed for determining recovery as they utilize an estimate. Overall, CSA was determined recover *S. aureus* from natural seawater adequately.

Morphologically, *S. aureus* colonies on CSA from natural waters were similar to those from seeded sterile waters. The non-target colonies on this medium were a variety of sizes (pinpoint to large, >3mm) and colors (white, yellow, green, blue, pink and purple; Figure 3.2). It was often difficult to distinguish target colonies on the membrane when total colony counts exceeded 100, which was seen in one-third of the CSA plates inoculated with 25 ml of seeded or natural seawater. In order to determine preliminary identification of these non-target colonies, gram stains were performed on a number of different types. All stains were either gram-positive cocci or rods.

To determine inhibitory ability of CSA for non-target cells, the number of total colonies (target and non-target) on CSA was compared to total counts on TSA. This was done with plates from four different seeded seawater samples and five different unseeded seawater samples. As a result, approximately 25.3% of total cells were recovered on CSA compared to TSA (Table 3.4), indicating an inhibition of 74.7% of cells. Variability in total cell recovery from natural seawater was also shown by the increased range in confidence interval, indicating that natural variances in samples are a factor, which was also apparent when *S. aureus* recovery was compared. Presumably, CSA is mostly inhibiting non-target cells as there was higher recovery from *S. aureus* seeded seawater samples (28.1%) compared to unseeded seawater samples (16.5%; Table 3.4). The total number of cells recovered on CSA, even with only 25 ml of sample filtered, was still high
enough to obstruct target colony recognition (Figure 3.2). Overall, CSA appears to have notable selectivity for *S. aureus* from seawater but needs improvement.

In summary, CSA adequately recovers *S. aureus* from natural seawater. Recovery of *S. aureus* on CSA from filter-sterilized waters was over 98% whereas from natural water recovery was less than 50%. This could be due to several factors including natural variances in water samples and variances in experimental preparations. In particular, the perceived recovery of *S. aureus* on CSA from seawater may be affected by the high levels of bacteria found in these waters. That is, the high level of non-target colonies recovered on CSA from seawater make it difficult to distinguish the target *S. aureus* colonies. Thus, the percent recovery of *S. aureus* from seawater on CSA may not be the ineffectiveness of the medium to grow *S. aureus* from seawater but rather failure of the investigator to identify target colonies. Hence, the selectivity of CSA needs improvement in order to enable operators to make distinctions between target *S. aureus* colonies and non-target colonies more easily when recovered from seawater.

3. **Addition of sodium azide to improve selectivity of CSA for *S. aureus* (Phase 3)**

Since the majority of non-target cells from seawater recovered on CSA were determined to be gram positive, an additional agent known to inhibit gram positives but not *S. aureus* was needed to improve the selectivity of this medium.

Sodium azide has been added to several media for isolation of *S. aureus* from natural waters (Borrego et al., 1983; Charoenca and Fujioka, 1998; Sato et al., 1995; and Stengren and Starzyk, 1984). The amount of azide used in previous media was typically 0.005%. It was important, however, to determine the appropriate percentage of sodium azide to add to CSA as the composition of this medium is unlike other media. The
appropriate percentage of sodium azide to add to CSA would be that which would improve selectivity for *S. aureus* from seawater without significantly affecting its recovery. This was addressed by comparing recovery amongst CSA modified by the addition of various concentrations of sodium azide. Comparisons were made using similar experimental procedures for assessing unmodified CSA (Phase two) but using counts from unmodified CSA as 100% recovery instead of TSA.

In the initial experiment, two types of seeded water (filter sterilized seawater and raw seawater) were seeded with *S. aureus* (ATCC 25923) and, using membrane filtration, inoculated onto CSA with 0.0025%, 0.003%, 0.0035%, and 0.004% sodium azide. Results from this experiment are summarized in Table 3.5. From these results, it was determined that 0.0035% was the most appropriate concentration of sodium azide to add to CSA. Recovery at this increment was slightly less than the cutoff value (0.004%) where recovery of *S. aureus* is good but total cell recovery has noticeably decreased. Since sodium azide is thought to be somewhat inhibitory to *S. aureus*, it was considered sensible to decrease the concentration added to the medium by a small fraction from the determined cutoff value.

Further investigation with numerous other experiments using different types of water and a broader range of sodium azide concentrations provided additional support for the previous conclusions (Table 3.6). Through these additional investigations it was again determined that the addition of 0.0035% sodium azide to CSA was most appropriate, providing good recovery of *S. aureus* (66.9%) while noticeably decreasing background (44.7% recovery of total colonies). Better values for recovery and inhibition are given in Table 3.6. These numbers, however, are compiled from a number of
experiments and therefore there is inherent variability between experiments that are not accounted for in the data. For example, it may appear that 0.004% is a more appropriate value than 0.0035% because a better recovery percentage for *S. aureus* and lower total colony recovery is presented. However, in this table it is the geometric mean of only three samples for 0.004% compared to 12 for 0.0035%. Also, the previous table (Table 3.5) presents a decreased recovery percentage for *S. aureus* from 0.004% (83.3%) than for 0.0035% (88.1%) and this data provides a more direct comparison between the two values as they are from the same experiment. Another issue with the values presented for the 0.0035% addition of sodium azide is the level of variability for *S. aureus* recovery as seen in the range for the confidence interval. This is likely an impact of inherent variability between experimental set up, including seed and media preparations. As mentioned previously, inherent differences in seed preparation can influence the amount of stress on cells and subsequent vulnerability to selective agents. For media preparation, small quantities were prepared because most of these experiments required only of few plates for each concentration of azide, which may have contributed to variability in selectivity due to inevitable errors in amounts distributed by pipettes and measured by weighing scales.

Qualitative data are not presented in any of the tables and is an important factor in assessing the amount of sodium azide to add to CSA. The addition of azide did affect the size and color of *S. aureus* colonies on CSA. With the addition of 0.005% sodium azide, colonies were pinpoint and considerably darker (purple) than on unmodified CSA. This made it especially difficult to distinguish target colonies on plates inoculated with raw seawater. Again, CSA with 0.0035% sodium azide was the best compromise between
altered morphology of target colonies and increased inhibition of non-target colonies. The addition of 0.0035% sodium azide did decrease the size of target colonies, by about one millimeter, and slightly darkened their mauve coloration.

As for unmodified CSA (Table 3.3), recovery of *S. aureus* from buffered water was lower than from filter-sterilized seawater (76.6% and 104%, Tables 3.7, and 30.8% and 99.1%, Table 3.8). The recovery from seeded raw seawater was lower than the sterile waters (41.5% and 104%, Table 3.7), which was also apparent for CSA (16.5% and 28.1%, Table 3.3). This latter point is troubling as the data in Table 3.7 is a comparison of CSA+A to CSA and data in Table 3.3 is CSA to TSA. These results imply that the true recovery (comparison with recovery on TSA) of CSA+A is 41.5% of 44.7%, which is 18.6%. However, the data for the recovery of CSA+A compared to TSA (Table 3.8) are similar to CSA compared to TSA (Table 3.3). Therefore, it may be differences in the natural water samples that attribute to the lower level of *S. aureus* recovery rather than a reflection of highly increased inhibition in CSA+A. In addition, as mentioned, inherent variability in media preparation may be a confounding factor. Recovery of *S. aureus* from filter-sterilized seawater on CSA+A compared to TSA is slightly higher than for CSA (99.1%, Table 3.8, and 98.6%, Table 3.3). The range for the confidence interval from CSA+A percent recovery is also less than for CSA, with values closer to the geometric mean (Tables 3.3 and 3.8). This indicates that CSA+A has a relatively similar recovery capability as CSA for *S. aureus* from seawater.

In summary, the addition of 0.0035% sodium azide improves the selectivity of CSA for *S. aureus* from seawater without drastically affecting the morphology or recovery efficiency. This modification does decrease recovery of *S. aureus* from water.
samples, with some experiments showing a significant reduction. However, when compared to other concentrations of sodium azide added to CSA, CSA with 0.0035% (CSA+A) provided the best fit for the desired criteria.

4. Comparison between recovery percentages for different strains of *S. aureus* from seeded water samples on CSA and CSA+A

As mentioned earlier, different preparation methods of *S. aureus* can be expected to apply different stresses to that population of bacteria and therefore *S. aureus* populations used for different experiments may vary, especially if used in different kinds of water samples. Such variability may also be attributed to the inherent level of stress a particular strain of *S. aureus* can endure. In order to assess if such a parameter is a factor in recoverability of *S. aureus* from seawater on CSA and CSA+A, different strains of *S. aureus* were used in the seeding experiments (see Materials and Methods section, Table 3.2). Table 3.9 is categorized by type of *S. aureus* strain used, and is a summary of results from experiments in which cells were added to water samples. These data suggest there was little difference in recovery values on CSA between ATCC reference strain and clinical and environmental strains (71.4% and 72.6% respectively). A difference between the types of *S. aureus* strains was seen on CSA+A, where recovery was lower for the reference strain (51.9%). The difference between recoveries on CSA+A and CSA for these two categories of *S. aureus* may be due to their difference in sample numbers (7 and 21 respectively). Indeed, if data for recovery of the reference strain on CSA are calculated using only the same experiments used to calculate recovery for CSA+A compared to CSA (n=7), the value decreases (54.6% with 95% C.I. = 38.6 to 102.8%). In contrast, if data for recovery of clinical and environmental strains on CSA are calculated
with corresponding experiments for CSA+A, the value increases for both CSA and CSA+A (n=4; CSA: TSA= 94.1% with 95% C.I. of 67.6 to 127%; CSA+A: CSA= 95.9% with 95% C.I. of 92.9 to 99.1%). Thus, when a more direct comparison is made, CSA and CSA+A have a decreased recovery for the reference strain compared to clinical and environmental strains. However, if a greater number of samples are included in the calculations, this difference is diminished for CSA and would likely be the same for CSA+A.

Overall, the data indicate that variability in recovery of *S. aureus* on CSA+A and CSA is not likely due to differences amongst strains of this pathogen.

5. **Evaluation of other additives for enhanced selectivity and recovery (Phase 4)**

Sodium azide is a strong selective agent and may be more detrimental to the target *S. aureus* cells than other selective agents. Thus, an assessment of other selective agents was needed to determine if some of these other selective agents might adequately inhibit non-target cells while improving recovery of *S. aureus* from seawater. In order to assess this, a number of selective agents (potassium thiocyanate, polymyxin B, and NaCl) were added to CSA and subjected to the same seeding experiments as was done for sodium azide modified CSA media. The results are summarized in Table 3.10. Only the addition of NaCl had some negative effect on total cell recovery on CSA (52.1%), but this was less than that seen when 0.0035% sodium azide was added (44.7%, Table 3.6). The addition of NaCl seemed to improve recovery of *S. aureus* for TSA (229%, Table 3.10), but few samples were tested to substantiate this conclusion.
As results from Phase 3 suggest, the recovery efficiency of CSA+A for *S. aureus* from seawater is rather poor (around 50% and highly variable). In addition, since seawater has poor nutrient content with a high concentration of cells, it is thought that *S. aureus* cells may be stressed and thus poorly recovered on such a highly selective medium. Therefore, enhancing agents that are known to promote growth for many bacterial cells were chosen to, possibly, improve recovery of *S. aureus* on CSA+A (see Material and Methods, Table 3.1).

Two enhancing agents, sodium pyruvate and glycine, were added to CSA in amounts used by Borrego et al. (1987) in their BFR-O medium (10 and 12 g/L respectively). The addition of pyruvate to CSA increased recovery of *S. aureus* in this medium (69.8% to 118%, Table 3.11), but significantly changed the morphology of *S. aureus* colonies making them appear pink instead of mauve. The use of glycine in CSA (C+G) did not seem to improve recovery of *S. aureus*, although the confidence interval was too broad to warrant this assessment (80.2%, 95% C.I. = 29.6% to 167%; Table 3.12). However, the morphology of *S. aureus* colonies on C+G did not significantly change compared to CSA, except for a slight increase in size. When glycine was added to CSA supplemented with sodium azide, both recovery (63.8% to 168%, Table 3.12) and morphology of *S. aureus* was improved. Though recovery does not appear to be improved for CSA with 0.001% sodium azide by the addition of glycine (C+0.001+G), the recovery for the other sodium azide modifications (C+0.0025+G and C+0.0035+G) of CSA were improved (Table 3.12). Interestingly, the addition of glycine also seemed to decrease the recovery of non-target cells (20.6% to 69.3%, Table 3.12).
It was stated previously that CSA with 0.0035% sodium azide (CSA+A) was the most suitable medium, from amongst the different media evaluated, for enhanced selectivity of *S. aureus* from seawater. This medium, however, had poor recovery of *S. aureus* from these waters. The data presented in this section show that the addition of glycine improved recovery of *S. aureus* in this modified medium (Table 3.12). When CSA with 0.0035% sodium azide and glycine (CSA+A+G) is compared to unmodified CSA (Table 3.13 and Figure 3.3), the recovery for *S. aureus* from water is 71.9%. This is an improvement from the recovery for *S. aureus* on CSA+A compared to CSA (66.9%, Table 3.7). Thus, the use of CSA+A+G is recommended over CSA+A for the selective recovery of *S. aureus* from seawater samples.

6. Specificity and selectivity of CSA containing media for *S. aureus*

The reliability of the differential component in the selected media was tested by confirming a number of isolates, target (mauve) and non-target (non-mauve), recovered from different marine water samples. Then, the numbers of isolates from CSA containing media that were false positive (mauve colonies not confirmed as *S. aureus*), true positive (mauve colonies confirmed as *S. aureus*), false negative (non-mauve colonies confirmed as *S. aureus*), and true negative (non-mauve colonies not confirmed as *S. aureus*) were used to calculate the specificities (ratio of true negatives amongst confirmed non- *S. aureus* isolates) and sensitivities (ratio of true positives amongst confirmed *S. aureus* isolates), as well as the positive predictive values (ratio of true positives amongst target isolates) and negative predictive values (ratio of true-negatives amongst non-target isolates), of the media (Table 3.14). The sensitivities of CSA, CSA+A, and CSA+A+G were good (>80%), indicating that most *S. aureus* form mauve
colonies. The positive predictive value for CSA+A+G, however, was poor (<70%). Thus, a greater number of false positives were identified on CSA+A+G. This poor value may be a reflection of the low number of isolates that were tested (n=86; Appendix C). The specificity of CSA was also good, but not so for CSA+A and CSA+A+G. This indicates that colonies that do not have the typical mauve morphology of *S. aureus* on both CSA+A and CSA+A+G may actually be *S. aureus*. Therefore, a greater number of false positives. The negative predictive value of CSA+A+G is much better than other media tested, which indicates this medium correctly identified most non-mauve isolates as non-*S. aureus*. Again, the low number of isolates from CSA+A+G that were tested may have skewed this value. Overall, these CSA containing media were relatively reliable at differentiating *S. aureus* from other organisms in seawater.

7. **Summary and recommendations**

The CSA medium recovers a high percentage (up to 100% with a mean of 71.8%) of *S. aureus* from seawater with a moderate degree of selectivity (up to 90% inhibition of non-target colonies, 75% mean) and can reliably identify this pathogen based on distinct colony morphology (medium sized, convex, opaque mauve colonies with diffuse mauve colored halo). Since the level and variety of bacteria in seawater is high, it was found that selectivity of CSA for *S. aureus* from seawater should be improved. Thus, modifications to CSA were made and this medium, CSA+A+G improved distinction of *S. aureus* colonies when there was a high level of non-target colonies in natural marine water samples (Figure 3.3).
Table 3.3. Percent recovery of *Staphylococcus aureus* from seeded water samples on CHROMagar™ Staph aureus (CSA) compared to tryptic soy agar (TSA).

<table>
<thead>
<tr>
<th>Water Type</th>
<th>No. of Samples (n)</th>
<th>Geometric Mean</th>
<th>Range</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL WATER</td>
<td>32</td>
<td>71.8</td>
<td>9.0-141</td>
<td>70.0 to 92.2</td>
</tr>
<tr>
<td>Buffered Water</td>
<td>10</td>
<td>78.3</td>
<td>50.0-111</td>
<td>67.7 to 94.3</td>
</tr>
<tr>
<td>Filter Sterilized Seawater</td>
<td>12</td>
<td>98.6</td>
<td>74.6-141</td>
<td>89.7 to 111</td>
</tr>
<tr>
<td>Seawater &amp; Brackish Water</td>
<td>10</td>
<td>44.7</td>
<td>9.0-136</td>
<td>33.1 to 82.9</td>
</tr>
</tbody>
</table>

Table 3.4. Percentage of total colony counts recovered from water samples on CHROMagar™ Staph aureus (CSA) compared to tryptic soy agar (TSA).

<table>
<thead>
<tr>
<th>Water Type</th>
<th>No. of Samples (n)</th>
<th>Geometric Mean</th>
<th>Range</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL WATER</td>
<td>5</td>
<td>25.3</td>
<td>16.5-60.0</td>
<td>9.6 to 41</td>
</tr>
<tr>
<td>Seeded Seawater &amp; Brackish Water</td>
<td>4</td>
<td>28.1</td>
<td>20.2-60.0</td>
<td>9.3 to 46.9</td>
</tr>
<tr>
<td>Un-seeded Seawater &amp; Brackish Water</td>
<td>1</td>
<td>16.5</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 3.5. Percent recovery of various modified CHROMagar™ Staph aureus (CSA) media compared to unmodified CSA, from a single experiment using two types of seeded water samples.

<table>
<thead>
<tr>
<th>% SODIUM AZIDE ADDED TO CSA</th>
<th>Cell Type</th>
<th>No. of Samples (n)</th>
<th>Arithmetic Mean</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0025</td>
<td>SA</td>
<td>2</td>
<td>99.4</td>
<td>±23.6</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>1</td>
<td>40.6</td>
<td>n/a</td>
</tr>
<tr>
<td>0.003</td>
<td>SA</td>
<td>2</td>
<td>93.3</td>
<td>±13.2</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>1</td>
<td>42.2</td>
<td>n/a</td>
</tr>
<tr>
<td>0.0035</td>
<td>SA</td>
<td>2</td>
<td>88.1</td>
<td>±17.8</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>1</td>
<td>34.9</td>
<td>n/a</td>
</tr>
<tr>
<td>0.004</td>
<td>SA</td>
<td>2</td>
<td>83.3</td>
<td>±16.4</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>1</td>
<td>31.4</td>
<td>n/a</td>
</tr>
</tbody>
</table>

SA – *S. aureus*; TOTAL – total bacteria
### Table 3.6. Percent recovery of various modified CHROMagar™ Staph aureus (CSA) media compared to unmodified CSA for bacteria from different water samples compiled from numerous experiments.

<table>
<thead>
<tr>
<th>Percent sodium azide added to CSA</th>
<th>0.0005</th>
<th>0.001</th>
<th>0.002</th>
<th>0.0025</th>
<th>0.003</th>
<th>0.0035</th>
<th>0.004</th>
<th>0.005</th>
<th>0.0075</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus No. of Samples (n)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Geometric Mean</td>
<td>114*</td>
<td>80.5*</td>
<td>59.7*</td>
<td>54.2</td>
<td>81.1</td>
<td>66.9b</td>
<td>77.3</td>
<td>37.6*</td>
<td>47.7</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>102 to 125</td>
<td>12.2 to 149</td>
<td>10.9 to 109</td>
<td>41.7 to 119</td>
<td>60.6 to 105</td>
<td>47.5 to 86.3</td>
<td>66.2 to 89.2</td>
<td>-28.9 to 145.5</td>
<td>n/a</td>
</tr>
<tr>
<td>No. of Samples (n)</td>
<td>1.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>1.00</td>
<td>6.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Geometric Mean</td>
<td>59.0</td>
<td>66.3*</td>
<td>40.8*</td>
<td>51.0*</td>
<td>42.2</td>
<td>46.7c</td>
<td>31.4</td>
<td>37.2</td>
<td></td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>20.9 to 112</td>
<td>33.1 to 48.5</td>
<td>28.0 to 74.0</td>
<td>30.9 to 65.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Arithmetic mean; * range = 4.5 – 114; b range = 23.3 – 139; c range = 26.3 – 84.3

### Table 3.7. Percent of *Staphylococcus aureus* recovered from various water samples on CHROMagar™ Staph aureus (CSA) with 0.0035% sodium azide (CSA+A) as compared to unmodified CSA.

<table>
<thead>
<tr>
<th>Water Type</th>
<th>No. of Samples (n)</th>
<th>Geometric Mean</th>
<th>Range</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL WATER</td>
<td>12</td>
<td>66.9</td>
<td>23.3-139</td>
<td>47.5 to 86.3</td>
</tr>
<tr>
<td>Seeded Buffered Water</td>
<td>3</td>
<td>76.6</td>
<td>57.7-98.6</td>
<td>55.2 to 102</td>
</tr>
<tr>
<td>Seeded Filter Sterilized Seawater</td>
<td>4</td>
<td>104</td>
<td>91.6-139</td>
<td>84.5 to 128</td>
</tr>
<tr>
<td>Seeded Seawater</td>
<td>4</td>
<td>41.5</td>
<td>23.3-97.1</td>
<td>15.6 to 82.6</td>
</tr>
<tr>
<td>Unseeded Seawater</td>
<td>1</td>
<td>51.3</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

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Table 3.8. Percent recovery for various strains of *Staphylococcus aureus* from seeded water samples on CHROMagar™ Staph aureus with 0.0035% sodium azide (CSA+A) compared to tryptic soy agar (TSA).

<table>
<thead>
<tr>
<th>Water Type</th>
<th>No. of Samples (n)</th>
<th>Geometric Mean</th>
<th>Range</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL WATER</td>
<td>7</td>
<td>69.7</td>
<td>19.4-138</td>
<td>53.9 to 140</td>
</tr>
<tr>
<td>Seeded Buffered Water</td>
<td>2</td>
<td>30.8</td>
<td>19.4-48.8</td>
<td>5.3 to 62.9</td>
</tr>
<tr>
<td>Seeded Filter Sterilized Seawater</td>
<td>5</td>
<td>99.1</td>
<td>70.7-138</td>
<td>79.8 to 122</td>
</tr>
</tbody>
</table>

Table 3.9. Comparative recovery percentages for two categories of *Staphylococcus aureus* strains, reference (ATCC 25933) vs. clinical and environmental (Materials and Methods: Table 2), on CHROMagar™ Staph aureus (CSA) and CSA with 0.0035% sodium azide (CSA+A).

<table>
<thead>
<tr>
<th>S. aureus type</th>
<th>Media Comparison</th>
<th>No. of Samples (n)</th>
<th>GMN</th>
<th>Range</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>CSA:TSA</td>
<td>21</td>
<td>71.4</td>
<td>9.0-136</td>
<td>66.9 to 94.7</td>
</tr>
<tr>
<td>Clinical and Environmental</td>
<td>CSA:TSA</td>
<td>11</td>
<td>72.6</td>
<td>15.0-141</td>
<td>62.2 to 101</td>
</tr>
<tr>
<td>Reference</td>
<td>CSA+A:CSA</td>
<td>7</td>
<td>51.9</td>
<td>23.3-139</td>
<td>31.6 to 95.8</td>
</tr>
<tr>
<td>Clinical and Environmental</td>
<td>CSA+A:CSA</td>
<td>6</td>
<td>79.4</td>
<td>51.3-98.6</td>
<td>64.8 to 99.4</td>
</tr>
</tbody>
</table>

GMN = geometric mean; TSA = Tryptic soy agar

Table 3.10. Percent recovery for *Staphylococcus aureus* (SA) and total bacteria (TOTAL) from seeded water samples on CHROMagar™ Staph aureus (C) modified with potassium thiocyanate (PT, number is g/L), polymyxin B (P) and salt (NaCl).

<table>
<thead>
<tr>
<th>Media comparison</th>
<th>Cell Type</th>
<th>No. of Samples (n)</th>
<th>Arithmetic Mean</th>
<th>Standard Deviation</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+5PT:CSA</td>
<td>SA</td>
<td>2</td>
<td>80.0</td>
<td>±1.9</td>
<td>±2.6</td>
</tr>
<tr>
<td>C+25PT:CSA</td>
<td>TOTAL</td>
<td>2</td>
<td>96.4</td>
<td>±4.0</td>
<td>±5.5</td>
</tr>
<tr>
<td>C+P:CSA</td>
<td>TOTAL</td>
<td>2</td>
<td>106.9</td>
<td>±24.2</td>
<td>±33.5</td>
</tr>
<tr>
<td>C+NaCl:CSA</td>
<td>SA</td>
<td>3</td>
<td>74.8</td>
<td>±4.9</td>
<td>±5.5</td>
</tr>
<tr>
<td>TSA+NaCl:TSA</td>
<td>TOTAL</td>
<td>1</td>
<td>52.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Geometric mean = 74.7*
Table 3.11. Effect on percent recovery of *Staphylococcus aureus* from seeded water samples for CHROMagar™ Staph aureus (C), C with 0.0035% sodium azide (C+A) and C with azide and glycine (C+A+G) after the addition of sodium pyruvate (SP, number is g/L) as compared to the medium specified after the colon.

<table>
<thead>
<tr>
<th>S. aureus</th>
<th>C+5SP: C</th>
<th>C+10SP: C</th>
<th>C+A+10SP: C+A</th>
<th>C+A+10SP: C+A+G</th>
<th>C+A+G+10SP: C+A+G</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Samples (n)</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Geometric Mean</td>
<td>118*</td>
<td>105*</td>
<td>69.8</td>
<td>161</td>
<td>337</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>±69.1</td>
<td>±34.5</td>
<td>60.8 to 79.4</td>
<td>48 to 530</td>
<td>-137 to 1305</td>
</tr>
<tr>
<td>TOTAL</td>
<td>140*</td>
<td>128*</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>No. of Samples (n)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Geometric Mean</td>
<td>140*</td>
<td>128*</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>±28</td>
<td>±15</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*Arithmetic mean; n.d. – no data; n/a – not applicable

Table 3.12. Effect on the addition of glycine (G) for percent recovery of *Staphylococcus aureus* (SA) and total colonies (TOTAL) from seeded water samples on CHROMagar™ Staph aureus (C) and C with sodium azide (C+%azide).

<table>
<thead>
<tr>
<th>Compared to same media without glycine</th>
<th>Cell Type</th>
<th>No. of Samples (n)</th>
<th>Geometric Mean</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+G</td>
<td>SA</td>
<td>4</td>
<td>80.2</td>
<td>29.6 to 167</td>
</tr>
<tr>
<td>C+G</td>
<td>TOTAL</td>
<td>1</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>C+0.001+G</td>
<td>SA</td>
<td>2</td>
<td>63.8</td>
<td>±31.7</td>
</tr>
<tr>
<td>C+0.001+G</td>
<td>TOTAL</td>
<td>1</td>
<td>20.6</td>
<td></td>
</tr>
<tr>
<td>C+0.002+G</td>
<td>SA</td>
<td>2</td>
<td>168</td>
<td>±35</td>
</tr>
<tr>
<td>C+0.002+G</td>
<td>TOTAL</td>
<td>1</td>
<td>26.4</td>
<td></td>
</tr>
<tr>
<td>C+0.0035+G</td>
<td>SA</td>
<td>9*</td>
<td>115</td>
<td>92.5 to 180</td>
</tr>
<tr>
<td>C+0.0035+G</td>
<td>TOTAL</td>
<td>4</td>
<td>69.3</td>
<td>27.4 to 136</td>
</tr>
</tbody>
</table>

* Range = 35.7 - 250
Table 3.13. Percent recovery for *Staphylococcus aureus* from seeded water samples on a few modified versions of CHROMagar™ Staph aureus (CSA) compared to unmodified CSA.

<table>
<thead>
<tr>
<th>Compared to CSA</th>
<th>No. of Samples (n)</th>
<th>Geometric Mean</th>
<th>Range</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+0.002+G</td>
<td>5</td>
<td>61.1</td>
<td>8.4 - 213</td>
<td>26.1 to 160</td>
</tr>
<tr>
<td>C+0.0035+G</td>
<td>6</td>
<td>71.9</td>
<td>22.2 - 340</td>
<td>14.9 to 203</td>
</tr>
<tr>
<td>C+0.005+G</td>
<td>1</td>
<td>318</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.14. Reliability of CHROMagar™ Staph aureus containing media to identify *Staphylococcus aureus* correctly from seawater based on distinct colony morphology, as determined by confirmation of 570 target and non-target isolates.

<table>
<thead>
<tr>
<th></th>
<th>SENSITIVITY</th>
<th>POS PRED</th>
<th>SPECIFICITY</th>
<th>NEG PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVERALL</td>
<td>86.4%</td>
<td>84.3%</td>
<td>85.0%</td>
<td>87.0%</td>
</tr>
<tr>
<td>CSA</td>
<td>86.6%</td>
<td>90.2%</td>
<td>87.7%</td>
<td>87.1%</td>
</tr>
<tr>
<td>CSA+A</td>
<td>93.8%</td>
<td>85.8%</td>
<td>51.6%</td>
<td>72.7%</td>
</tr>
<tr>
<td>CSA+A+G</td>
<td>92.6%</td>
<td>64.1%</td>
<td>76.3%</td>
<td>95.7%</td>
</tr>
</tbody>
</table>

CSA = CHROMagar™ Staph aureus  
CSA+A = CSA with 0.0035% sodium azide,  
CSA+A+G = CSA+A with 12g/L glycine.
Figure 3.2. CHROMagar\textsuperscript{TM} Staph aureus (CSA) plated with 25 milliliter (bottom plate) and CSA with 0.0035\% sodium azide plated with 100milliliter (top plate) of seawater collected from Ala Moana Beach Park.

Figure 3.3. 100 ml of seawater concentrated onto a membrane filter and plated on CHROMagar\textsuperscript{TM} Staph aureus (CSA, top) and CSA with azide and glycine (CSA+A+G, bottom); note the reduction in background and visibility of mauve colonies (circled in red) on CSA+A+G.
A. Objectives

Isolates recovered from CHROMagar™ Staph aureus (CSA) plates can be determined as methicillin-resistant *Staphylococcus aureus* (MRSA). However, in order to determine the quantity of MRSA present, all presumptive colonies of *S. aureus* must be recovered for testing. This method is too tedious and not feasible for routine evaluations. If one is interested in determining concentrations, presence, or recovering several isolates of MRSA from seawater samples, more selective media or methods are required. The objective for the next part of the study was to develop methods that would differentiate MRSA among the *S. aureus* isolated from natural marine recreational waters. In order to meet this objective, evaluations of methods for direct detection, screening and enrichment were done. All presumptive MRSA isolates recovered from water samples were confirmed using approved methods.

B. Materials and Methods

1. Evaluation of CSA supplemented with oxacillin for direct detection of MRSA

   Previous investigations using clinical specimens found that CSA supplemented with oxacillin was useful for the direct detection of MRSA. In order to determine if CSA supplemented with oxacillin may be useful in enhancing the recovery of MRSA from seawater, a range of concentrations (1, 2, and 4 μg/ml) of this antibiotic were added and then these supplemented media subjected to the same recovery and selectivity tests as was done for the unmodified media (Chapter Three, Materials and Methods). In addition,
a range of concentrations of sodium azide were added along with oxacillin at different concentrations and similarly tested for recovery and selectivity.

2. Evaluation of a screening method

Screening involves the analysis of a group of bacteria in order to determine susceptibility to a particular antibiotic. Screening methods using specific media (usually containing a particular antibiotic) to test isolates for resistance, determined by the presence of growth, prior to more rigorous susceptibility tests or even prior to confirmation of the organism have been employed by diagnostic laboratories as a means to more rapidly determine appropriate treatment regimens for patients. In this study, a screening method for determining oxacillin resistance amongst isolates recovered from seawater that utilized CSA was developed. In order to determine the appropriate concentration of oxacillin to add to CSA (1μg/ml, 2μg/ml or 4μg/ml), known MRSA, methicillin intermediate S. aureus (MISA) and methicillin susceptible S. aureus (MSSA) isolates as well as several presumptive S. aureus isolates were inoculated onto these media. This was done by obtaining a small amount of fresh growth and making a single streak onto each medium. If mauve growth appeared it was isolated and tested for resistance by disk diffusion. The concentration of oxacillin that gave the least amount of false positives or negatives was then employed in further screening efforts.

3. Evaluation of an enrichment broth

Enrichment allows low numbers of bacteria that cannot be detected by standard methods to grow to some high level so they can be detected. However, an enrichment step usually precludes enumeration of original concentration. Previous investigators have also suggested the inclusion of an enrichment step in MRSA screening protocols.
(Apfalter et al., 2002; Davis, 1997; Gardam, et al., 2001). In this study, the numbers of MRSA in the environment were apparently low and an enrichment step may enhance the recovery of MRSA. General broth media would provide the best recovery of these pathogens. However, such an enrichment method would also grow several non-target cells. Thus, several parameters to improve selectivity of general media were tested (Table 4.1). Such testing was done by preparing samples with very low concentrations (eg. 1 CFU/100ml) of known MRSA isolates, filtering high volumes (up to 1 liter) of these samples onto membranes, inoculating the membranes into 100 milliliter aliquots of test enrichment broth and then incubating at particular temperature (37°C, 40°C, or 45°C). The resulting turbidity of the growth within the different test broths was compared (- = no turbidity, +/- = slight turbidity, etc. up to +++ = very turbid with flocculation) and 0.1 ml of these enrichments plated onto CSA with 4 μg/ml oxacillin (CSA+O). After incubation, the overall abundance of mauve colonies and non-target colonies on these spread plates was compared by their overall abundance in order to determine which broth effectively recovered low levels of MRSA from seawater.

4. Methods to determine resistance of S. aureus isolates to methicillin

A summary of the methods used to determine methicillin resistance amongst S. aureus isolates is given in Figure 4.1. The disk diffusion method uses standardized procedures to detect antimicrobial susceptibility or resistance to an antibiotic based on zones of inhibition (ZOI). The ZOI is the area of no bacterial growth around an antimicrobial impregnated disk that has been placed on a well inoculated agar plate. To detect resistance to methicillin the antibiotic oxacillin is used because it has a more stable molecular structure than methicillin that employs the same mechanism as methicillin
against cell wall formation within gram-positive cells. The diameter of the ZOI can be correlated to the minimum inhibitory concentrations (MICs) due to the differential diffusion of the antimicrobial agent into the agar. However, MICs cannot be directly inferred from ZOIs, only interpretive categories (susceptible, intermediate, and resistant) can be assigned to isolates tested with the disk diffusion method. In this study, initial testing for antibiotic sensitivity to oxacillin was performed using standard methods for antimicrobial disk susceptibility (NCCLS, 2003).

Susceptibility test methods, such as disc diffusion and agar screening, are often inaccurate due to affects of inoculum size, incubation time and temperature, media, pH, salt concentrations and other factors (NCCLS, 2003; Tenover, et al., 1999). Direct detection of the \textit{mecA} gene has been considered the gold standard in the determination of methicillin-resistance because of its accuracy but this method is tedious and expensive. To confirm resistance after disk diffusion testing, a latex test that detects the low-affinity penicillin binding protein (PBP2'), which is encoded by the \textit{mecA} gene, was used in this study (Oxoid). Several reports have demonstrated this test to be highly sensitive and specific for the detection of methicillin-resistance in staphylococci isolates as compared to detection of \textit{mecA} gene by polymerase-chain reactions (Felten et al, 2002; Louie, et al, 2000; Smyth et al, 2001; vanLeeuwen, et al, 1999). In this test, latex particles with monoclonal antibody against PBP2' react specifically with methicillin-resistant staphylococci to cause visible agglutination. This test entails using a fresh culture to prepare a very turbid suspension of cells in extraction reagent and then heating above 95°C for several minutes. Once cooled, a second extraction reagent is added, mixed, and then centrifuged to separate the extracted PBP2'. A small amount of supernatant is
mixed with test and control latex on a test card. The test card is rocked by hand and agglutination, if positive, is apparent within three minutes.

5. Strains of *Staphylococcus aureus*

Several different strains of *S. aureus* (Table 4.2) were used when preparing samples to assay CSA with oxacillin not only to assess if inherent differences within a *S. aureus* population will affect the selectivity or recoverability of the media but, also, to determine what concentration of oxacillin in CSA will suitably select for MRSA. Some of the prepared cell suspensions in buffered water, filter-sterilized seawater, and/or seawater were a mix of strains. This was done to mimic more accurately the assemblage of *S. aureus* in water, in which several types of *S. aureus* are likely to be present due to their variety of sources (e.g. different humans). Although adding various strains to one sample will eliminate the ability to detect variances in recovery ability of the tested media due to strain differences, it will still allow comparison of overall recoverability of the tested media for *S. aureus* from the prepared water samples.
Table 4.1. Mechanisms reported to improve selective recovery of methicillin-resistant *Staphylococcus aureus* (MRSA).

<table>
<thead>
<tr>
<th>Target Organism</th>
<th>Selective Agents</th>
<th>Enhancement of Growth</th>
<th>Other selective/differential parameters</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA</td>
<td>Oxacillin</td>
<td>Enrichment in broth with high nutrient content prior to plating on selective medium</td>
<td>Tolerates high salt concentrations (delete to recover from seawater). Temperature for growth: 18-40°C</td>
<td>Merlino et al., 2000; Kluytmans et al., 2002</td>
</tr>
</tbody>
</table>

Table 4.2. Characteristics of strains of *Staphylococcus aureus* used to seed seawater for media tests of CHROMagar™ Staph aureus with oxacillin (1, 2, and 4 μg/ml).

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Source</th>
<th>Type</th>
<th>Concentration of Oxacillin Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25923</td>
<td>Standard stock</td>
<td>S</td>
<td>All concentrations</td>
</tr>
<tr>
<td>6/1</td>
<td>Clinical</td>
<td>R</td>
<td>All concentrations</td>
</tr>
<tr>
<td>6/4</td>
<td>Clinical</td>
<td>R</td>
<td>All concentrations</td>
</tr>
<tr>
<td>10-1</td>
<td>Environment</td>
<td>R</td>
<td>All concentrations</td>
</tr>
<tr>
<td>8-11</td>
<td>Environment</td>
<td>S</td>
<td>All concentrations</td>
</tr>
<tr>
<td>8-82</td>
<td>Environment</td>
<td>R</td>
<td>All concentrations</td>
</tr>
<tr>
<td>12-F</td>
<td>Environment</td>
<td>I</td>
<td>All concentrations</td>
</tr>
<tr>
<td>14-15</td>
<td>Environment</td>
<td>S</td>
<td>All concentrations</td>
</tr>
<tr>
<td>14-64</td>
<td>Environment</td>
<td>S</td>
<td>All concentrations</td>
</tr>
<tr>
<td>17-76</td>
<td>Environment</td>
<td>R</td>
<td>4 μg/ml</td>
</tr>
</tbody>
</table>

Type = oxacillin susceptible (S) or oxacillin resistant (R); Environment = recovered from seawater sample (ID for these corresponds to Experiment # - Isolate # in Appendix D); Clinical = recovered from patient specimens and identified at commercial laboratory.
Figure 4.1. Flow chart of resistance testing after confirmation as *Staphylococcus aureus* of isolates recovered from seawater using CHROMagar™ Staph aureus containing media (TSA = tryptic soy agar, R = resistant, I = intermediate resistance, S = susceptible; + = positive, - = negative; MRSA = methicillin-resistant *S. aureus*, MSSA = methicillin-susceptible *S. aureus*)
C. Results and Discussion

1. Oxacillin supplemented CSA for direct detection of MRSA from water samples

Although methods using CSA to detect MRSA directly from clinical samples have been reported, currently no such method for marine recreational waters has been published. In this study, CSA supplemented with oxacillin (1 to 4 μg/ml) was evaluated using membrane filtration methods for quantitative recovery of MRSA from marine recreational waters. Because of these studies, it was found that the addition of oxacillin appears to increase selectivity of CSA for MRSA but may not effectively recover this target organism. Table 4.3 provides a summary of the experiments using CSA supplemented with oxacillin. When 4 μg/ml of oxacillin was added to CSA, the recovery of MRSA from water samples decreased by about 50% compared to the addition of 1 or 2 μg/ml of oxacillin (Table 4.3). However, there was a high level of variability amongst the tests for this medium, as evidenced by the confidence intervals. Data presented in this table are not a direct comparison between results from individual experiments, but rather the mean results from several different experiments. When recovery percentages are compared directly, per experiment, the recovery percentage of only a clinical strain of MRSA (6/1) from filter sterilized seawater is significantly lower on CSA with 4 μg/ml than with 2 μg/ml (4.1% and 22.0%, Table 4.4). Thus, variability between preparation of cell suspension, strain types, media preparations, seawater samples, and other sources of variability may be affecting the recovery percentages of MRSA on CSA with oxacillin. Interestingly, these data also show that 1 μg/ml of oxacillin in CSA has a lower recovery percentage (50.0%) than 2 μg/ml (73.6%) for the mix of MRSA isolates in buffered water. It is logical to assume that a higher concentration of an inhibitory substance, such as
oxacillin, would also have a higher level of inhibition and, therefore, lower recovery.

This set of data, however, contradicts this assumption. In a later experiment using seeded buffered water, a similar effect was seen, where an increase in oxacillin concentrations added to CSA resulted in a 30% increase of recovery percentage (61.4% to 91.4%, Table 4.5). In yet another experiment that employed seeded seawater, there was no effect on recovery when oxacillin concentrations were increased (17.5%, Table 4.5). Being that these data represent results from only a few experiments, the difference could very well be due to inherent variances in the experimental methods. Overall, there was a slight reduction in recovery of MRSA when CSA with oxacillin was compared to CSA without supplementation. This is one indication that MRSA cells may be stressed and thus not be able to produce enough resistant proteins for direct recovery on media with oxacillin. Thus, direct quantitative recovery of MRSA from beach waters on CSA with oxacillin may not be a reliable method. However, given the constricted number of evaluations completed, the only conclusion that is validated is that there was no credible difference in recovery of MRSA when the amount of oxacillin in CSA was increased to 4μg/ml. CSA with 4μg/ml of oxacillin (CSA+O) was reported previously as useful in detecting MRSA from clinical specimens (Klutymans et al., 2002; Merlino et al., 2000). Results from this study provide no firm evidence that CSA+O can be applied to the recovery of MRSA from seawater.

As was done for CSA, CSA+A and CSA+A+G, the reliability of CSA+O to correctly identify S. aureus by distinct colony morphology was tested by confirming a number of isolates, target and non-target, recovered from different marine water samples. The CSA+O medium was found to have poor sensitivity (44.4 %) as well as a poor
positive predictive value (57.1 %), meaning its ability to correctly identify \textit{S. aureus} was not reliable. The specificity and negative predictive value of CSA+O was determined to be very good (94.5% and 91.2%), which indicates that colonies which do not have the typical mauve morphology of \textit{S. aureus} are not likely to be \textit{S. aureus}. However, the low number of isolates from CSA+O that were tested may have skewed these values and, although further analyses were not performed, they would be needed before any valid conclusions about the reliability of CSA+O to identify \textit{S. aureus} directly could be made.

2. Addition of azide to oxacillin supplemented CSA to improve selectivity for MRSA from seawater

Since the addition of azide to CSA proved useful in improving this medium’s selectivity for \textit{S. aureus} from seawater, a similar supplementation to CSA with oxacillin may improve selectivity for MRSA. In order to assess this hypothesis, several evaluations of using different combinations of oxacillin and sodium azide concentrations in CSA were performed.

In one experiment, which used a mix of \textit{S. aureus} strains to seed buffered water the results showed that the addition of oxacillin to CSA with sodium azide had minor effect on recovery, when compared to unmodified CSA (63.2 to 80.7%, Table 4.5). In this same experiment, the addition of 0.0025% and 0.005% sodium azide to CSA supplemented with oxacillin had approximately the same effect on recovery (63.2 to 69.3%). A higher concentration of azide would be expected to decrease recovery, as was seen previously, but the similarity in recovery percentages between a higher and lower concentration of added sodium azide indicates there was not such an effect when oxacillin was also present. However, the recovery percentages for CSA with 0.0035%
sodium azide and oxacillin (78.1% to 80.7%) were better than when 0.0025% (63.2% to 69.3%) or 0.005% (63.2% to 68.4%) of sodium azide was added. A recovery percentage of 80.7% from buffered water was determined for CSA with 1 μg/ml of oxacillin and 0.0035% sodium azide, which is comparable to the recovery for CSA with 0.0035% sodium azide that was determined previously (88.1%, Chapter Two, Table 3.5), but an increase in oxacillin to 4μg/ml slightly lowered recovery to 78.1%. However, also apparent in this data is that, overall, the recovery percentages were similar when an increased amount of oxacillin was added to CSA with the same amount of sodium azide. Thus, an increase in oxacillin did not appear to have as much of an affect as changes in sodium azide concentrations.

When the same mix of *S. aureus* cells used to seed buffered water was seeded into natural seawater, the recovery on CSA with oxacillin and azide was very poor (7.5% to 40.0%; Table 4.5). Also, when the concentration of oxacillin was increased (2μg/ml to 4μg/ml) in CSA with 0.0035% or 0.005% sodium azide, half as many MRSA cells were recovered (15% to 7.5% and 40% to 12.5%, respectively). Therefore, an increase in oxacillin in CSA with sodium azide had a greater inhibitory effect on MRSA recovered from natural seawater than what was found when these cells were recovered from buffered water. What caused this discrepancy in recovery between water types is not clear, but perhaps this is partly due to recognition of target colonies amongst various other colonies. In which, cells that form very small colonies can still be distinguished when recovered from sterile waters that were seeded with target cells; whereas, distinction of such small colonies is significantly reduced when they are amongst other background colonies that were recovered from the un-sterilized, natural seawater. In this
natural seawater experiment, there was also a slight increase (about 22.5%) in recovery of MRSA when a higher concentration of azide (0.0025% more) was added to CSA with oxacillin, suggesting that azide may aide CSA in selecting for the rarely occurring MRSA cells.

To see more clearly the effects of sodium azide versus oxacillin on recovery of *S. aureus* from water for CSA, it is best to compare one modification of CSA at a time. When CSA with oxacillin and sodium azide was compared to CSA with only oxacillin, it was shown that in CSA with 1μg/ml oxacillin the addition of sodium azide appeared to increase recovery (>100%, Table 4.6). Amongst these comparisons, there was a decrease in recovery (about 30%) when azide was added to CSA with 2 μg/ml of oxacillin. Suggesting that the combination of azide with a higher concentration of oxacillin is more inhibitory to *S. aureus* than when a lower amount of oxacillin is used. When an opposing comparison was done, CSA with oxacillin and 0.0035% sodium azide compared to CSA with sodium azide, nearly half as many cells were recovered when a greater concentration of oxacillin was added (1200% to 600%, Table 4.6). Previous data showed that the recovery of MRSA from seawater on CSA with 0.0035% azide and 4mcg/ml oxacillin (CSA+A+O) compared to CSA (Table 4.5) was 16.2% and here (Table 4.6) when compared to CSA+A the recovery is 600.0%. This indicates that most of the inhibition may be attributed to the addition of sodium azide rather than oxacillin and this assumption correlates well with previous results, where recovery of MRSA on CSA+O compared to CSA was around 80%.

Overall, these data suggest there is little effect on MRSA recovery when oxacillin is added to CSA compared to unmodified CSA. The addition of azide to CSA with
oxacillin may have some inhibitory effect on cells, but this addition may still be useful if it can improve selectivity for MRSA when significant amounts of other bacterial cells are present.

The addition of azide was shown previously to be useful in recovering *S. aureus* from seawater on CSA by significantly inhibiting background colonies which made identification and isolation of target colonies easier (Chapter 3). When the addition of azide to CSA with oxacillin was evaluated, it was found that the recovery percentage of total colonies from seeded seawater for CSA with 0.0035% azide and 2 mcg/ml of oxacillin (CSA+A+2O) compared to CSA with 2 mcg/ml oxacillin (CSA+2O) was nearly 100%, indicating that the addition of azide did not improve selectivity of CSA+2O (Table 4.7). Within these same results, only about one third of the total cells were recovered when CSA+A+2O was compared to CSA+A; indicating that oxacillin will increase the selectivity of CSA+A. Also, results from experiments mentioned in Chapter 3 indicated that of total cells from seawater on CSA+A compared to CSA was 44.7%, whereas the results in Table 4.7 show CSA with 2 μg/ml of oxacillin (CSA+2O) compared to CSA was only 25.4%. Thus, from these experiments, oxacillin had a greater inhibition than azide for total (mostly non-target) cells recovered on CSA from seawater. Data in Table 4.7 also show that if the concentration of oxacillin is increased in CSA+A, there is no significant decrease in total cell recovery (42.8% to 24.8%) that would indicate an increase in selectivity. Additionally, an increase in azide concentration to CSA with oxacillin did not noticeably improve inhibitory quality (i.e. C+A+2OX = 24.8% and C+0.005+2Ox = 39.9%). Since the inhibition to MRSA that sodium azide imposes is great, without a substantial increase in the inhibition of background bacteria compared to
the inhibition that oxacillin already generates, the validity of using sodium azide in addition to oxacillin is questionable.

In conclusion, when the addition of sodium azide to CSA with oxacillin was tested, the results indicated that sodium azide was of little use for the enhanced selectivity of MRSA from seawater on CSA with oxacillin.

3. Adjusted incubation period for MRSA recovery on oxacillin supplemented CSA

Since MRSA recovery was poor on CSA with sodium azide and/or oxacillin (<60%) it was thought that perhaps a longer incubation time would improve both recovery and colony size. In an experiment using a suspension of MRSA cells in buffered water and membrane filtration methods, it was found that all of the CSA media with additional selective substances had better recovery after 48 hours incubation than at 24 hours (Figure 4.2). Without the addition of sodium azide and/or oxacillin the recovery of MRSA on CSA was the same at 24 and 48 hours; this was also the case for the general medium TSA. This indicates that MRSA, and perhaps all *S. aureus* strains, grow slower on CSA in the presence of sodium azide and/or oxacillin. Klutymans et al. (2002) also found CSA+O to have increased sensitivity for MRSA isolates after 48 hours incubation as opposed to 24 hours. In their study, prolonged incubation also increased the number of false-positives, which may account for the poor sensitivity value for CSA+O established in this study (44.4 %).

4. Evaluation of a screening method

The growth of several strains of MSSA and MRSA on CSA with different amounts of oxacillin added revealed that a lower concentration was not largely inhibitory to MRSA or MISA strains and not adequately selective against MSSA strains (Figure
Although the clinical MRSA strain 6/4 was sensitive to an increase in oxacillin, a concentration of 1\(\mu\)g/ml still allowed growth of a known MSSA strain (ATCC 25923).

Since 1\(\mu\)g/ml of oxacillin did not adequately select resistant strains of \textit{S. aureus} from susceptible strains, concentrations of 2 \(\mu\)g/ml and 4 \(\mu\)g/ml of oxacillin in CSA were used to screen presumptive \textit{S. aureus} colonies recovered on CSA from seawater. Of the 45 isolates recovered on CSA with oxacillin and later confirmed as \textit{S. aureus}, 19 were recovered from CSA+2O and 26 from CSA+O (Table 4.8). Of the 19 isolates recovered on CSA+2O, all were determined to be susceptible to oxacillin by disk diffusion tests. Whereas, over a quarter of the 26 recovered on CSA+O were determined by disk diffusion tests to have some resistance to oxacillin. These results suggest that the use of 4 \(\mu\)g/ml of oxacillin in CSA will increase selectivity for resistant strains over the use of 2 \(\mu\)g/ml of oxacillin. A similar result was reported by Klutymans et al. (2002) who investigated the ability of CSA+O and another medium, which had 2 \(\mu\)g/ml oxacillin, to discriminate between MSSA and MRSA isolates. These investigators found that CSA+O had no false positives (growth of MSSA isolates) while the other medium with a lower oxacillin concentration had a rate of 0.8% and 5.3% false positives after 24 and 48 hours incubation respectively. Thus, CSA with 4 \(\mu\)g/ml of oxacillin (CSA+O) is recommended for use as a screening agar to recover MRSA from seawater.

When CSA+O was used to screen confirmed \textit{S. aureus} isolates that had been recovered from seawater beforehand, it was found that 63 isolates exhibited at least a little growth. These 63 isolates were then tested for oxacillin resistance using disk diffusion methods. The results reveal that only a few of the isolates that displayed moderate (colony formation) to good growth (extensive colony formation) exhibited any
degree of resistance to oxacillin (4.0% to 22.7%, Table 4.9). This indicates that when using CSA+O to screen for oxacillin resistance in *S. aureus* isolates, only those isolates that demonstrate at least a moderate amount of the typical mauve colored growth on CSA+O should undergo further resistance tests (see Figure 4.3 for example of typical mauve coloration).

In summary, the addition of 4mcg/ml of oxacillin to CSA (CSA+O) was determined to perform best as a screening medium, compared to lower concentrations of oxacillin in CSA. Screening with CSA+O may be performed either by directly inoculating presumptive colonies from primary isolation plates (spot plating) or inoculating previously recovered and confirmed *S. aureus* isolates (screening), incubating 36-48 hours, and using isolates with at least moderate growth to additionally test for resistance with disk diffusion and latex agglutination.

5. Evaluation of an enrichment broth

Several enrichment methods have been employed in various microbiological fields to improve the recovery of stressed or minority cells. Since *S. aureus*, especially MRSA, were likely to be limited in numbers, enrichment methods were evaluated for their use in improving the detection and subsequent recovery of MRSA from seawater.

Initial experiments addressed the use of temperature and duration of incubation as selective mechanisms for MRSA from seawater and employing nutrient broth as the enrichment medium. These experiments used an environmental MRSA strain (TF10-1) to seed filter sterilized seawater and natural seawater (1-12 CFU/L), incubation of inoculated broths at temperatures of 37°C, 41°C and 50°C, and followed by loop inoculation onto CSA+O. Turbidity of these enrichment broths, determined by visual
evaluation, revealed that incubation at 41°C will allow growth of *S. aureus* (turbidity in seed inoculated broth; Table 4.10). Though turbidity of unseeded seawater had little difference between 37°C and 41°C, a visibly significant decrease in the turbidity at 50°C suggests that an increase in temperature will have some inhibition for bacteria in seawater that are unable to grow at higher temperatures. Since 41°C can support growth of *S. aureus*, this slight increase in temperature may enhance selectivity of other seawater enrichments. Turbidity of pure seed enrichments indicated that an incubation of 18 hours was adequate to recover *S. aureus*. While a longer incubation would complicate recovery of the target organism by allowing increased growth of non-target organisms as indicated by the increased turbidity of pure seawater enrichment broths after 24 hours compared to 18 hours of incubation time. Growth on CSA+O inoculated with enrichments showed that a few target colonies can be recovered from seeded seawater, though non-target colonies usually outnumber target colonies (Table 4.11). Confirmation results from of a few of the target colonies on CSA+O revealed that MRSA was recovered from enrichment broths that were incubated at 41°C and 45°C (Table 4.12). It was determined from the experiments that low concentrations of MRSA in seawater can be recovered using nutrient broth for enrichment. These methods entailed using nutrient broth inoculated with filter concentrated sample, incubated at 41°C overnight and this enrichment inoculated on CSA+O. With these stated methods, it was found that *S. aureus* could be recovered from seawater while limiting recovery of most other bacteria. There were a few types of background bacteria, however, that were still readily recovered, which might interfere with recovery of target bacteria.
Later experiments assessed if additives (oxacillin and polymyxin B) to nutrient broth could improve selection for MRSA from seawater. The first of these incorporated oxacillin at increasing concentrations (2µg/ml, 4µg/ml, and 6µg/ml) with overnight incubation at 37°C and 41°C. A reference strain of *S. aureus* (ATCC 25923, final concentration ~2x10³ CFU/liter) and a clinical MRSA isolate (6/1, final concentration ~70 CFU/liter) were used to seed seawater, one liter of the cell suspension filtered and inoculated into the test broth, and, after incubation, spread plating (0.1ml) of enrichment onto CSA+O. A single target mauve colony was found on CSA+O inoculated with an overnight enrichment in nutrient broth with 4 µg/ml of oxacillin (NB+4O) after 48 hours incubation (Table 4.13). The most common colonies recovered were blue, and these were previously determined to be catalase negative, gram-positive cocci (Table 4.12). Interestingly, an increase in oxacillin to enrichment broth seemed to increase recovery of blue colonies, especially at 6 µg/ml of oxacillin (Table 4.13). Thus, the addition of oxacillin to nutrient broth increased selectivity for resistant cells but not specifically *S. aureus*. Confirmation tests for several isolates recovered from CSA+O spread plates after 24 hours incubation revealed not all MRSA had typical mauve morphology, as all of the purply pink colonies tested were determined to be *S. aureus* by agglutination (Table 4.14). As discussed previously (Section 3), growth of MRSA on CSA may be slower in the presence of oxacillin, which may also affect the metabolism of the chromogenic substance, decreasing the degree of mauve coloration. In addition, Klutymans et al. (2002) found that the sensitivity of CSA+O increased with longer incubation period, which may have been due to a slower development of a more typical mauve coloration amongst the target colonies. A few confirmed *S. aureus* isolates were tested for
resistance to oxacillin by disk diffusion and all were determined to be resistant strains, indicating oxacillin prevented growth of MSSA seeded in seawater samples (Table 4.14).

The next experiment assessed the usefulness of polymyxin B (3 to 30 µg/ml) addition to nutrient broth for selective recovery of MRSA from seawater. This experiment was similar to oxacillin experiments, except only a clinical MRSA strain was used (final concentration ~70 CFU/100 ml) and buffered water (final concentration ~230 CFU/100 ml), broths were inoculated with five milliliters of cell suspensions, and all the inoculated broths were incubated overnight at 37°C instead of 41°C. Results indicated that the addition of polymyxin B to nutrient broth instead of oxacillin does not seem to improve the recovery of MRSA cells from seawater. The addition of increasing amounts of polymyxin B appeared to reduce recovery of MRSA cells, as evidenced by the lower broth turbidity and subsequent recovery of colonies on CSA+O and CSA from enrichments that were inoculated with the buffered water cell suspension (Table 4.15). In addition, the increase in concentrations of polymyxin B to nutrient broth did not significantly reduce the non-target colonies recovered on CSA+O or CSA from enrichments inoculated with either seeded or unseeded seawater (Table 4.15). The concentrations (3 to 30 µg/ml) of polymyxin B added in this experiment were low compared to what had been used in other media (Borrego et al., 1985) and higher concentrations (eg. 75 µg/ml) may have sufficiently reduced the level of background. Higher concentrations, however, may also reduce recovery of MRSA since, in this experiment, the recovery of MRSA cells from buffered water decreased when concentrations of in the enrichment broth increased.
Given the results from these few experiments, the addition of oxacillin to NB is more useful than polymyxin B to enrich recovery of MRSA from seawater. Oxacillin suppresses the growth of cells that are susceptible, limiting amount of growth in the broth. Although, it appears to have no prejudice for resistant *S. aureus* and allows other resistant cells to grow. These non-target cells were mostly catalase negative, gram-positive cocci. Inhibition of these organisms should be considered in future development of enrichment methods for MRSA recovery from seawater. Since the purply pink colonies were determined to be mostly MRSA and NB+2O had a greater recovery for this type of colony than higher concentrations of oxacillin, this broth is suggested. The following enrichment methods are recommended: NB+2O incubated overnight at 40°C, subsequent plating onto CSA+O with 48 hour incubation at 37°C, and isolation of colonies with any degree of mauve coloration (Figure 4.4).

6. Oxacillin susceptibility testing

A total of thirty isolates were tested for production of PBP2' using the latex agglutination (Appendix D). Five isolates tested were not confirmed to be *S. aureus* prior to testing and one of these was not tested with disk diffusion. Of those tested, eighteen were definitively positive, two had weak agglutination reactions, and ten were considered negative. These results correlate well with disk diffusion results, except for two. One isolate that was determined to be negative for PBP2' had previously tested positive by the same test. No clear reason for this discrepancy exists, but storage conditions (refrigerated in broth) may weaken the expression of the *mec* gene. Another isolate was positive with PBP2' agglutination and classified susceptible to oxacillin by disk diffusion. This may be due to a lower expression of the *mec* gene, where PBP2' agglutination is more
sensitive than disk diffusion which relies on a higher expression. Thus, the disk diffusion method is relatively accurate at classifying levels of resistance to oxacillin amongst *S. aureus* isolates. The use of PBP2' latex agglutination test is still recommended, as it is simple and provides an added level of confidence for the diagnosis of resistance in these pathogens.

7. **Summary and recommendations for selective recovery of MRSA from seawater**

No substantial reduction in recovery of MRSA from buffered water on CHROMagar Staph aureus plus 0.0035% sodium azide and 2 μg/ml oxacillin (CSA+A+O) compared to CSA without these additives was evident. Only about 60% of the background non-target colonies are recovered on CSA+A+O when compared to CSA. Thus, this additional inhibition of background may justify the use of CSA+A+O even though there is also some inhibition of the target cells when recovered from seawater. An increased level of background inhibition may allow a larger volume of sample to be filtered without the interference of background colonies on recognition and isolation of suspected MRSA. However, the use of sodium azide seems to be a significant factor in the inhibition of the target cells on CSA and the use of this additive in conjunction with oxacillin for recovery of MRSA should not be for quantitative recovery. Therefore, it is recommended that CSA+A+O be used to isolate MRSA from large volumes (~500ml) of seawater by filtration. In addition, CSA plus 4 μg/ml of oxacillin (CSA+O) used to isolate MRSA from smaller volumes (~100ml) of seawater by filtration, to screen suspected *S. aureus* colonies from CSA, CSA+A, or CSA+A+G for resistance, and to screen confirmed *S. aureus* isolates for oxacillin resistance.
Table 4.3. Cumulative data for percent recovery of methicillin-resistant *Staphylococcus aureus* from various seeded water samples on CHROMagar™ Staph aureus (CSA) with oxacillin.

<table>
<thead>
<tr>
<th>Compared to TSA</th>
<th>Compared to CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Samples (n)</td>
</tr>
<tr>
<td>CSA+1µg/ml Oxacillin</td>
<td>1</td>
</tr>
<tr>
<td>CSA+2µg/ml Oxacillin</td>
<td>3</td>
</tr>
<tr>
<td>CSA+4µg/ml Oxacillin</td>
<td>3</td>
</tr>
</tbody>
</table>

N/A = not applicable, TSA = Tryptic soy agar, C.I. = confidence interval (a statistical measurement)

Table 4.4. Percent recovery of different methicillin-resistant *Staphylococcus aureus* strains from two types of seeded water. Percentage determined by comparing recovery on test media to the reference media, plated during each of the individual experiments, a direct comparison.

<table>
<thead>
<tr>
<th>Water Type</th>
<th>MRSA strain</th>
<th>Compared to TSA</th>
<th>Compared to CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter sterilized seawater</td>
<td>6/1 (clinical)</td>
<td>n.d.</td>
<td>22.0</td>
</tr>
<tr>
<td>Filter sterilized seawater</td>
<td>6/4 (clinical)</td>
<td>n.d.</td>
<td>88.9</td>
</tr>
<tr>
<td>Buffered water</td>
<td>Mix* (clinical and environmental)</td>
<td>50.0</td>
<td>73.6</td>
</tr>
</tbody>
</table>

* All of the following isolates added: 6/1 (clinical), 6/4 (clinical), and TF10-1 (environmental); n.d. = no data; TSA = Tryptic soy agar, CSA = CHROMagar™ Staph aureus, C+1OX = CSA with 1µg/ml oxacillin, C+2OX = CSA with 2µg/ml oxacillin, C+4OX = CSA with 4µg/ml oxacillin
Table 4.5. Percent recovery of methicillin-resistant *Staphylococcus aureus* on modified CHROMagar™ Staph aureus (CSA) media compared to unmodified CSA from individual experiments using different types of water as seeding solution.

<table>
<thead>
<tr>
<th>Water Type</th>
<th>MRSA strain</th>
<th>CS with oxacillin</th>
<th>Percent Recovery</th>
<th>CS with azide and oxacillin</th>
<th>Percent Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffered Water</td>
<td>Mix*</td>
<td>μg/ml of oxacillin</td>
<td></td>
<td>μg/ml of oxacillin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% sodium azide</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>61.4</td>
<td>1</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>91.4</td>
<td>2</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td>4</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>2</td>
<td>0.005</td>
</tr>
<tr>
<td>Seeded Seawater</td>
<td>Mix*</td>
<td></td>
<td></td>
<td>1</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>0.005</td>
</tr>
<tr>
<td>Buffered water</td>
<td>17-76</td>
<td>4</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(environmental)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All of the following isolates added: 6/1 (clinical), 6/4 (clinical), and TF10-1 (environmental)
Table 4.6. Recovery percentages for methicillin-resistant *Staphylococcus aureus* on CHROMagar™ Staph aureus with multiple modifications compared to a single modification, from experiments using different types of waters as seeding solutions.

<table>
<thead>
<tr>
<th>Water Type</th>
<th>MRSA strain</th>
<th>µg/ml of oxacillin</th>
<th>% sodium azide</th>
<th>Percent Recovery</th>
<th>µg/ml of oxacillin</th>
<th>% sodium azide</th>
<th>Percent Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffered Water</td>
<td>Mix*</td>
<td>1</td>
<td>0.0025</td>
<td>113</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.0025</td>
<td>69.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.0035</td>
<td>131</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.005</td>
<td>103</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.005</td>
<td>75.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seeded Seawater</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0.0035</td>
<td>1200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>0.0035</td>
<td>600</td>
</tr>
</tbody>
</table>

* 6/1 (clinical), 6/4 (clinical), & TF10-1 (environmental); CSA = CHROMagar Staph aureus™

Table 4.7. Percentage of total colonies recovered from seeded and unseeded seawater on various media containing CHROMagar™ Staph aureus compared to other media.

<table>
<thead>
<tr>
<th>Test media</th>
<th>C+2Ox</th>
<th>C+A</th>
<th>CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+A+2Ox</td>
<td>97.8</td>
<td>29.5</td>
<td>24.8</td>
</tr>
<tr>
<td>C+A+1Ox</td>
<td>82.0</td>
<td>42.8</td>
<td></td>
</tr>
<tr>
<td>C+A+4Ox</td>
<td>79.4</td>
<td>39.7</td>
<td></td>
</tr>
<tr>
<td>C+2Ox</td>
<td></td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td>C+0.005+1Ox</td>
<td></td>
<td>51.1</td>
<td></td>
</tr>
<tr>
<td>C+0.005+2Ox</td>
<td></td>
<td>39.9</td>
<td></td>
</tr>
<tr>
<td>C+0.0025+1Ox</td>
<td></td>
<td>70.5</td>
<td></td>
</tr>
<tr>
<td>C+0.0025+2Ox</td>
<td></td>
<td>53.8</td>
<td></td>
</tr>
</tbody>
</table>

C+2OX = CSA with 2µg/ml oxacillin, C+A = CSA with 0.0035% sodium azide, CSA = CHROMagar Staph aureus™, C+A+2OX = C+A with 2µg/ml oxacillin, C+A+1Ox = C+A with 1µg/ml oxacillin, C+A+4OX = C+A with 4µg/ml oxacillin, C+0.005+1OX = CSA with 0.005% sodium azide and 1µg/ml oxacillin, C+0.005+2OX = CSA with 0.005% sodium azide and 2µg/ml oxacillin, C+0.0025+1OX = CSA with 0.0025% sodium azide and 1µg/ml oxacillin, C+0.0025+2OX = CSA with 0.0025% sodium azide and 2µg/ml oxacillin
Table 4.8. Disk diffusion test results of isolates recovered from oxacillin supplemented CHROMagar™ Staph aureus (CSA) when used to screen for oxacillin resistance amongst presumptive Staphylococcus aureus colonies (mauve) recovered from seawater on various CSA containing media.

<table>
<thead>
<tr>
<th></th>
<th>C+20 (N=19)</th>
<th>C+40 (N=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA</td>
<td></td>
<td>23.1%</td>
</tr>
<tr>
<td>MISA</td>
<td></td>
<td>3.8%</td>
</tr>
<tr>
<td>MSSA</td>
<td>100%</td>
<td>73.1%</td>
</tr>
</tbody>
</table>

C+2O = CSA with 2 μg/ml oxacillin; C+4O = CSA with 4μg/ml oxacillin; N = number of isolates

Table 4.9. Disk diffusion test results of confirmed Staphylococcus aureus isolates in relation to level of growth on screening agar (CHROMagar™ Staph aureus with 4μg/ml oxacillin).

<table>
<thead>
<tr>
<th>No. Tested</th>
<th>Good Growth</th>
<th>Moderate Growth</th>
<th>Some Growth</th>
<th>Very Little Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant</td>
<td>5 (22.7%)</td>
<td>1 (4.0%)</td>
<td>6</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>5 (22.7%)</td>
<td>1 (4.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Susceptible</td>
<td>12 (54.6%)</td>
<td>23 (92.0%)</td>
<td>6 (100%)</td>
<td>10 (100%)</td>
</tr>
</tbody>
</table>

Table 4.10. Relative turbidity of nutrient broths inoculated with various seeded (methicillin-resistant Staphylococcus aureus) and unseeded waters that were incubated at various temperatures and times.

<table>
<thead>
<tr>
<th>18 hours</th>
<th>seed</th>
<th>sea</th>
<th>Seed sea</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>41°C</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>50°C</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>24 hours</td>
<td>seed</td>
<td>sea</td>
<td>Seed sea</td>
</tr>
<tr>
<td>37°C</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41°C</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>45°C</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>50°C</td>
<td>+/-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>55°C</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36 hours (+12 hours @ 37°C)</td>
<td>seed</td>
<td>sea</td>
<td>Seed sea</td>
</tr>
<tr>
<td>45°C</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50°C</td>
<td>+/-</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>55°C</td>
<td>-</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

-= no turbidity; +/- = slightly turbid; + = turbid; ++ = highly turbid; and +++ = very turbid with flocculation; seed = seeded buffered water; sea = unseeded raw seawater; seed sea = seeded raw seawater
Table 4.11. Description of type and amount of bacterial growth on spread plates of CHROMagar™ Staph aureus with 4µg/ml of oxacillin inoculated with enrichment broth cultures that were incubated at stated temperature and for indicated time, in hours (Broth).

<table>
<thead>
<tr>
<th>Broth</th>
<th>Sea</th>
<th>Seeded Sea</th>
<th>Seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C-18 h</td>
<td>Numerous blue, few small whitish, few erose pinkish, and 2 small pinkish purple</td>
<td>Several blue</td>
<td>n.g.</td>
</tr>
<tr>
<td>37°C - 24 h</td>
<td>N/A</td>
<td>N/A</td>
<td>n.g.</td>
</tr>
<tr>
<td>41°C - 18 h</td>
<td>Few blue</td>
<td>Numerous blue, few white, some erose mauve and small pinkish</td>
<td>n.g.</td>
</tr>
<tr>
<td>41°C - 24 h</td>
<td>several dark purple, turquoise, and dark lavender</td>
<td>n.g.</td>
<td>1 mauve, 2 small purply pink</td>
</tr>
<tr>
<td>41°C - 24 h</td>
<td>n.g.</td>
<td></td>
<td>1 mauve</td>
</tr>
<tr>
<td>45°C - 26 h (+12 h @ 37°C)</td>
<td>N/A</td>
<td>N/A</td>
<td>Few mauve, numerous pinkish purple, several purply pink</td>
</tr>
<tr>
<td>55°C</td>
<td>n.g.</td>
<td>n.g.</td>
<td>n.g.</td>
</tr>
</tbody>
</table>

Sea = seawater, Seeded Sea = seawater with added methicillin-resistant *Staphylococcus aureus* (MRSA) cells, Seed = buffered water with added MRSA cells; N/A = not applicable; n.g. = no growth

Table 4.12. Confirmation tests of a few isolates recovered from CHROMagar™ Staph aureus with 4µg/ml of oxacillin (CSA+O) plated with overnight enrichments that were incubated at stated temperature.

<table>
<thead>
<tr>
<th>Source</th>
<th>CSA+O morphology</th>
<th>Gram Stain</th>
<th>Catalase</th>
<th>Latex</th>
<th>Oxacillin Disk (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea 41°C</td>
<td>Blue</td>
<td>+ cocci</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed sea 41°C</td>
<td>Mauve</td>
<td>+ cocci</td>
<td>+</td>
<td>+</td>
<td>S (17)</td>
</tr>
<tr>
<td>Seed sea 41°C</td>
<td>Mauve</td>
<td>+ cocci</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed sea 41°C</td>
<td>Pinkish</td>
<td>+ cocci</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed sea 41°C</td>
<td>Mauve</td>
<td>+ cocci</td>
<td>+</td>
<td>+</td>
<td>R (7)</td>
</tr>
<tr>
<td>Seed 41°C</td>
<td>Mauve</td>
<td>+ cocci</td>
<td>+</td>
<td>+</td>
<td>R (6)</td>
</tr>
<tr>
<td>Seed 41°C</td>
<td>Purply pink</td>
<td>+ cocci</td>
<td>+</td>
<td>-</td>
<td>R (6)</td>
</tr>
<tr>
<td>Seed 45°C</td>
<td>Mauve</td>
<td>+ cocci</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Sea = seawater, Seeded Sea = seawater with added methicillin-resistant *Staphylococcus aureus* (MRSA) cells, Seed = buffered water with added MRSA cells; + = positive, - = negative; S = susceptible, R = resistant
Table 4.13. Description of CHROMagar™ Staph aureus with 4μg/ml of oxacillin spread plates inoculated with enrichment broths of seawater with added methicillin-resistant Staphylococcus aureus cells.

<table>
<thead>
<tr>
<th>Enrichment Broth</th>
<th>Hours of Incubation for Spread Plates</th>
<th>Description of colonies (number of colonies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB</td>
<td>24</td>
<td>All blue colonies (~100)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Dark and light blue (~150), light pink (~150), white</td>
</tr>
<tr>
<td>NB+2O</td>
<td>24</td>
<td>Blue (&gt;1000), small/pinpoint purply pink (&gt;20)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Blue, large white (~50), medium cream/pink (~75)</td>
</tr>
<tr>
<td>NB+40</td>
<td>24</td>
<td>Blue (&gt;1000), light purply pink (~10)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Blue, large white (~10), medium cream/pink (~15), medium mauve with halo (1)</td>
</tr>
<tr>
<td>NB+6O</td>
<td>24</td>
<td>Millions of pinpoint blue</td>
</tr>
</tbody>
</table>

NB = nutrient broth, NB+2O = NB with 2 μg/ml oxacillin, NB+4O = NB with 4 μg/ml oxacillin, NB+6O = NB with 6 μg/ml oxacillin

Table 4.14. Confirmation tests for isolates recovered from enrichments of seawater with added methicillin-resistant Staphylococcus aureus MRSA cells using CHROMagar™ Staph aureus with 4μg/ml of oxacillin (CSA+O) as spread plate medium.

<table>
<thead>
<tr>
<th>Broth Source</th>
<th>CSA+O morphology</th>
<th>Catalase</th>
<th>Latex</th>
<th>Coagulase (tube)</th>
<th>Oxacillin Disk (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB+2O (24)</td>
<td>Purply pink</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>NB+2O (24)</td>
<td>Purply pink</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>NB+2O (48)</td>
<td>Pink</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NB+2O (48)</td>
<td>Pink</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>NB+4O (24)</td>
<td>Purply pink</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>NB+4O (24)</td>
<td>Purply pink</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NB+4O (48)</td>
<td>White</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NB+4O (48)</td>
<td>Mauve</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6</td>
</tr>
</tbody>
</table>

NB+2O = NB with 2 μg/ml oxacillin, NB+4O = NB with 4 μg/ml oxacillin; number in parenthesis under “Broth Source” is hours of incubation of spread plate
Table 4.15. Comparisons between enrichment broth turbidity and subsequent recovery on CHROMagar™ Staph aureus with 4μg/ml of oxacillin (CSA+O) and CSA after spread plating.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>NB+1.5P</th>
<th>NB+3P</th>
<th>NB+30P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Turbidity</td>
<td>CSA+O</td>
<td>CSA</td>
</tr>
<tr>
<td>Seeded PBS</td>
<td>High</td>
<td>Many mauve</td>
<td>N.G.</td>
</tr>
<tr>
<td>Seeded Seawater</td>
<td>Some</td>
<td>Several mauve, many pink</td>
<td>Several peachy mauve</td>
</tr>
<tr>
<td>Unseeded Seawater</td>
<td>High to moderate</td>
<td>Many blue, several lt. pink</td>
<td>High to moderate</td>
</tr>
</tbody>
</table>

NB+1.5P = nutrient broth with 1.5 μg/ml of polymyxin B, NB+3P = NB with 3 μg/ml of polymyxin B, NB+30P = NB with 30 μg/ml of polymyxin B; N.G. = no growth, It. = light; seeded water had added methicillin-resistant *Staphylococcus aureus*
Figure 4.2. Comparison between average numbers of methicillin-resistant *Staphylococcus aureus* colonies recovered from seeded buffered water on various media tested after 24 and 48 hours of incubation (TSA – tryptic soy agar, CSA – CHROMagar™ Staph aureus, C+1 – CSA with 1µg/ml oxacillin, C+2 - CSA with 2µg/ml oxacillin, C+0.0025+1 – CSA with 0.0025% sodium azide and 1µg/ml oxacillin, C+0.0025+2 - CSA with 0.0025% sodium azide and 2µg/ml oxacillin, C+0.0035+1 - CSA with 0.0035% sodium azide and 1µg/ml oxacillin, C+0.0035+4 - CSA with 0.0035% sodium azide and 4µg/ml oxacillin, C+0.005+1 - CSA with 0.005% sodium azide and 1µg/ml oxacillin, C+0.005+2 - CSA with 0.005% sodium azide and 2µg/ml oxacillin).
Figure 4.3. Relative growth values (0.5 = very little, 1.0 = some, 2.0 = moderate, 3.0 = good) on the various media tested using known methicillin-susceptible Staphylococcus aureus {Reference: SA; Environmental: #64, 15, 11, 704}, methicillin-intermediate S. aureus (Environmental: F) and methicillin-resistant S. aureus {Clinical: 6/1, 6/4; Environmental: #1, and 82} strains. Note: 15 had intermediate resistance from disk diffusion tests (TSA - tryptic soy agar, CSA - CHROMagar\textsuperscript{TM} Staph aureus, C+1 - CSA with 1\mu g/ml oxacillin, C+2 - CSA with 2\mu g/ml oxacillin, C+4 - CSA with 4\mu g/ml oxacillin).

Figure 4.4. Colony colors of presumptive methicillin-resistant Staphylococcus aureus on CHROMagar\textsuperscript{TM} Staph aureus with 4\mu g/ml oxacillin inoculated with enrichment broth (mauve = pinky purple).
CHAPTER FIVE

Assessment of *Staphylococcus aureus* in Marine Waters Using CSA Based Media and Membrane Filtration Methods

A. Objectives

In this part of the study the objective was to evaluate CSA based media and membrane filtration method to enumerate *S. aureus* bacteria in marine recreational environments. This examination was performed in order to further evaluate various media’s selectivity for *S. aureus*, to observe the persistence and survival ability of *S. aureus* in marine water, to determine possible environmental sources of *S. aureus* in marine waters and to correlate *S. aureus* concentrations in water samples with the number of swimmers.

B. Materials and Methods

1. Sample collection and assay

Marine water samples were collected from the surface (approximately two feet deep) in sterile polyethylene containers (Nalgene) and immediately stored in an ice-chest. All water samples were transported to the laboratory and processed within six hours of collection. Appropriate dilutions of the samples, prepared in buffered water, were filtered through 0.45 μm membrane filters (Gn-6 Gelman or MF-Millipore) using the membrane filtration technique. Filters were then placed on selected media (see part two) and incubated 24 – 48 hours at 37°C; resulting colonies enumerated and selected colonies were further isolated for purification and confirmation. All samples were analyzed with
diluted and undiluted sub-samples with the mean value of the countable plates, calculated as CFU/100ml, recorded as the final concentration. A flow chart of the membrane filtration process employed is given in Figure 5.1.

Sand samples were collected into sterile Nalgene bottles, immediately stored in an ice-chest and analyzed in the laboratory within six hours of collection. Sand samples were weighed (10 g) and diluted 1:10 in sterile buffered water (95 ml). This solution was then "hand-shaken" for five minutes and then serially diluted in sterile buffered water using 1:10 increments. Primarily the resulting supernatant was tested, since sand settles rapidly. Spread plate or membrane filtration techniques were then used with appropriate amounts of the dilutions to inoculate the media. A portion of the fresh sand sample was weighed and oven-dried and the ratio from the weight of the oven-dried sand to the fresh sand used to calculate CFU/gram of oven-dried sand.

2. Media

Several types of media were utilized to recovery bacteria from seawater. CSA, CSA+A and/or CSA+A+G were utilized as selective/differential media to directly recover and enumerate S. aureus from marine recreational waters. The CSA base used in these media was obtained from CHROMagar™ laboratories (DRG distributors). Selected methods, based on earlier evaluations, using nutrient broth with oxacillin were used as a supplement to enhance recovery of MRSA isolates. For detection of Enterococci, the standard indicator bacteria for marine recreational waters (7 CFU/100ml in Hawaii), membrane filtration methods using modified Enterococci agar (mE) media with subsequent plating onto esculin iron agar (EIA) media were employed.
3. **Confirmation tests**

In order to establish the sensitivity and specificity of the different CSA containing media for *S. aureus* recovered from marine water, several mauve and non-mauve colonies were isolated and subjected to various confirmation tests. The protocol of biochemical tests discussed in Chapter one were also used in this phase of the study. In addition, a DNA probe specific to *S. aureus* RNA (Accuprobe, Gene-Probe) and an automated ribotyping instrument (Riboprinter®, DuPont) were employed.

4. **Sampling sites**

Seventy three samples of marine recreational waters and three samples of brackish water taken from 16 different sites (Table 5.1; Fig. 5.2) around the island of Oahu were analyzed. Ten sampling sites were located in the southern section of the island (including brackish water site), 2 on the north coast, 2 on the east coast, and 2 on the western side. Several of these sites were previously classified by Charoenca and Fujioka (1989) into “High Staph” and “Low Staph” beaches based on the levels of total staphylococci and *S. aureus*. In their study, the cut-off point for defining “Low Staph” beaches was less than 100 staphylococci/100 ml or 10 *S. aureus*/100 ml. In this study, samples were taken from a few of the sites listed, with alternation, on a monthly to bi-monthly basis at peak hours of bather usage (10 a.m. - 3 p.m.). Standard methods were used to collect and transport surface water samples from these sites (Greenburg et al., 1992). All samples were analyzed for *S. aureus* and total colony counts (Gram positives) on CSA. A few samples were also analyzed for total Enterococci using mE media.
5. Survival of *Staphylococcus aureus* in seawater and sand

Experiments were performed to determine whether *S. aureus* could multiply or survive prolonged periods in seawater. The first experiment entailed seeding low concentrations of *S. aureus* cells into filter sterilized seawater collected from Sans Souci beach. The types of *S. aureus* cells used in this experiment included an MSSA strain (ATCC #25923), two clinical MRSA strains (Diagnostic Labs: 6/1 and 6/4) and one MRSA strain recovered from marine water (TF10-1). The suspension of cells in seawater was then left at room temperature and ambient light (fluorescent with natural light from windows or only fluorescent lighting; fluorescent light only during work day hours, approximately 8 a.m. to 6 p.m.) with enough stirring from magnetic stir bar to form a whirlpool in the flask. Levels of *S. aureus* were measured (on TSA, CSA, CSA+A+G and CSA+O media) at time zero and then every six hours for 24 hours.

The next experiment measured levels of *S. aureus* in sand and nutrient enriched seawater over a period of two weeks. This entailed adding low levels of MRSA to sterilized seawater (seed). The seawater was sterilized by filtering (0.22µm) and then autoclaving. The sterilized seawater was then used to prepare the samples, which included an un-inoculated control, an un-enriched control, and two that were enriched with peptone (0.0005% and 0.005%). Prepared sand by autoclaving and adding enough diluted seed to reach a 10% moisture content. The control was an un-inoculated sub-sample of sand prepared with sterile buffered water. Moisture content was maintained by maintaining sample weight (minus sub-sampling amounts) with buffered water. Sub-samples from the seawater and sand samples were taken at regular intervals (Table 5.2) and tested for recoverable cells using CSA and TSA media.
Table 5.1. Identification and characterization of sampling sites (coastal and recreational beaches on Oahu, Figure 5.2).

<table>
<thead>
<tr>
<th>Oahu Location</th>
<th>Bather Density</th>
<th>Major Bather Type</th>
<th>Circulation</th>
<th>Nutrient Inputs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>Local</td>
<td>Tourist</td>
</tr>
<tr>
<td><strong>SOUTH SHORE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sand Island+</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Ala Moana *</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala Wai Canal</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magic Island</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duke Kahanamoku</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Kuhio Beach Park*</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Sans Souci</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waialae Beach Park+</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wailupe Beach Park+</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hanauma Bay State Underwater Park*</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WINDWARD SIDE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waimanalo Beach Park</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kailua Beach Park</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><strong>NORTH SHORE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waimea Bay</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Haleiwa Ali'i Beach Park</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><strong>LEEWARD SIDE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Makaha Beach Park</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ewa Beach Park</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Ala Wai Canal is not a recreational beach and is not designated for swimming
* = classified as “High Staph” by Charoenca and Fujioka (1993); ^a = vulnerable to run-off; + = classified as non-swimming beach in this study; X = present; High bather density = on average >25; Low bather density = on average <25; Local = resident, Tourist = visitor or non-resident
Table 5.2. Timeline for water and sand sub-sampling during *Staphylococcus aureus* survival experiment.

<table>
<thead>
<tr>
<th>TIME</th>
<th>CONTROL</th>
<th>NO PEPTONE</th>
<th>0.0005% PEPTONE</th>
<th>0.005% PEPTONE</th>
<th>CONTROL</th>
<th>TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>6</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>36</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>72</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>125</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
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</tr>
<tr>
<td>14 d</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CONTROL = filter-sterilized seawater without added *S. aureus*, NO PEPTONE = filter-sterilized seawater with added *S. aureus*, 0.0005% PEPTONE = amount of peptone added to filter-sterilized seawater with added *S. aureus*, 0.005% PEPTONE = amount of peptone added to filter-sterilized seawater with added *S. aureus*, SAND = sand with added *S. aureus* in filter-sterilized seawater; X = sub-sample taken; time is in hours except for 14 d. which is number of days.
Water Sample

Dilute in 225 ml phosphate buffer

Filter through sterile 0.45 µm membrane filter

Place filter in broth

Incubate aerobically at 42°C for 18 hours

Spread plate 0.1 ml onto CSA+O

Incubate aerobically at 37°C for 36-48 hours

Enumerate mauve

Mauve growth (MRSA) isolated

CONFIRMATION AND RESISTANCE TESTING

Figure 5.1. Flow chart of method to enumerate Staphylococcus aureus and recover methicillin-resistant S. aureus from marine waters (CSA = CHROMagar™ Staph aureus, CSA+A = CSA with 0.0035% sodium azide, CSA+A+G = CSA+A with 12g/L glycine, CSA+O = CSA with 4µg/ml oxacillin, NB+O = nutrient broth with 2µg/ml oxacillin).
Figure 5.2. Location of sampling sites on the island of Oahu.
C. Results and Discussion

1. Overview

During the 13 month study (July 2003 to August 2004) 75 water samples were collected, 25% during winter (November – April) and 75% during summer (May – October), as well as 10 sand samples that were collected during the summer. Table 5.3 provides the number of water samples that were collected for each site. All water and sand samples were collected and analyzed with CSA. In addition, 25 samples were analyzed with CSA+A and 42 with CSA+A+G (none of the samples were analyzed with both these modified versions of CSA). In this report, swimming beaches refers to those beaches where >10 persons were consistently present at the time of sampling, those sites which did not fit this criteria were classified as non-swimming (Materials and Methods section, Table 5.1).

2. Presence and distribution of Staphylococcus aureus in the coastal areas of Oahu

The presence of S. aureus was established in 92.0% of samples (n=75) and at 87.5% of the sites studied (Appendix A). Overall, the numbers of S. aureus ranged from 0 to 124 CFU/100 ml with an arithmetic mean of 22 CFU/100ml. Therefore, S. aureus was detected at the majority of the sites studied. This frequency of S. aureus at popular marine recreational beaches was also observed by Yoshpe-Purer and Golderman (1987) in which 91% of their samples taken during highest bathing periods were positive for S. aureus.

High mean concentrations (>8 CFU/100 ml) of S. aureus were established at several sites including Kuhio and Sans Souci, whose range of concentrations also included extremely high values (124 and 102 CFU/100 ml respectively; Table 5.3). High
concentrations of *S. aureus* (≥10 CFU/100ml, suggested cut-off level by Charoenca (1991), were found primarily at crowded beaches (Kuhio, Sans Souci, Hanauma Bay, Duke Kahanamoku, Magic Island, Ala Moana, Ewa and Kailua) indicating bathers as an important source of *S. aureus* in seawater. However, a low concentration (3 CFU/100 ml) of *S. aureus* was recovered from a crowded beach (Waimea Bay). Waimea Bay, a popular beach on the northern shore of Oahu, is relatively more exposed to the sea than the other popular beach sites tested and also receives run-off from a nearby stream. Most of the other sites have some sort of enclosure be it outer reef, fabricated barrier, or direction facing open sea, which can hinder seawater transport. Waimea, even though a bay, is devoid of such protection and thus relatively exposed, making water transport much greater at this site. This site also has a steep beach slope compared to the other areas tested and therefore there is typically fewer small children in the water due to the strong currents and undertow that is indicative of steeply sloped beaches. Ewa beach is also a relatively exposed, steeply sloped beach which is less popular than Waimea, yet this site had high concentrations of *S. aureus* from both the samples taken. Ewa Beach Park not only receives canal discharge but is also proximal to the entrance of Pearl Harbor. Therefore, these waterways may have influenced *S. aureus* counts. However, no tests were been done to determine if Pearl Harbor or the nearby canal are possible sources of *S. aureus*. Overall, *S. aureus* was recovered more frequently from swimming beaches (95.7%) and at higher concentrations (average of 24 CFU/100ml) than non-swimming beaches which had a lower frequency of recovery (20.0%) and lower concentrations (average of 7 CFU/100ml, Table 5.4). These results support the hypothesis that the source of *S. aureus* is from direct human use, that some people release *S. aureus* into
recreational waters, that these pathogens remain viable in water, that there is a risk for swimmers who become physically exposed to this water, and that this risk is higher at heavily used beaches. These findings coincide with those of other studies including Cheung et al. (1991) whom observed an association between bather density and levels of staphylococci at two popular beach sites in Hong Kong. As well, Papadakis et al. (1997) found a significant correlation between number of swimmers and *S. aureus* levels in the water during their study of two recreational beaches in Greece. Charoenca and Fujioka (1993) also found a strong correlation with *S. aureus* concentrations and number of beach users present at the time of sampling in their investigation of Hawaiian marine recreational waters.

Methicillin-resistant *S. aureus* isolates were only recovered from highly visited beaches as well (Kuhio, Sans Souci, Hanauma Bay, and Ala Moana, Table 5.3), which also infers humans as the primary source. Upon characterization, approximately 6% of the *S. aureus* isolates that were tested were resistant to methicillin. Another 3% were found to have intermediate resistance (MISA). MRSA currently causes the most serious staphylococcus infections in Hawaii and its presence in marine beach waters is the first evidence needed to infer that swimming at some beaches may result in its transmission.

3. *Staphylococcus aureus* counts according to season and fecal indicators

*Staphylococcus aureus* was recovered often, few days with no *S. aureus* recovered, in both summer (May – October) and winter (November – April) but at greater levels in summer (Geometric Mean for “All days” in Table 5.5). In this data, Ala Moana does not appear to have seasonal variation but the range of *S. aureus* levels in summer and winter (1-58 CFU/100 ml and 3-16 CFU/100 ml) indicates that greater levels are recoverable in
summer. However, given the data provided, this seasonality of \textit{S. aureus} levels in marine waters is not evident from non-swimming sites. Although the number of people present is similar at the most popular sites in summer and winter, summer weather conditions may provoke an increase in an individual’s amount of time spent bathing. If indeed \textit{S. aureus} is shed from bathers, then an increase in the amount of bathing in summer would then correlate with the increase in \textit{S. aureus} levels that were detected in this study.

Ten marine water samples were analyzed for levels of enterococci. This group of bacteria is the only fecal indicator approved by the Environmental Protection Agency (EPA) for use in assessing seawater for public health risks to sewage borne infections. In Hawaii, the acceptable level of enterococci in these waters is $<7$ CFU/100 ml from a geometric mean of 5 samples taken over a month’s time, whereas a single sample cannot exceed 100 CFU/100 ml. Six of the samples tested exceeded 7 CFU/100ml (Table 5.6). Two sampling sites, Wailupe and Ala Wai directly receive stream water discharge. The Wailupe sample was taken just after a rainfall event and was clearly affected by the discharge from the nearby stream. Kailua is a beach that is near a stream outlet and Kuhio is an enclosed beach with an opening to the ocean that is close to a discharge point from a storm drain. Enterococci have been shown to survive and even proliferate in the soils of Hawaii, partly due to its tropical habitat, which is favorable to their maintenance (Roll and Fujioka, 1997). Contamination at Hanauma Bay was discussed by Oshiro and Fujioka (1995) where they found high levels of fecal indicators in the sand. Incidentally, they found that the sand at this site contained a good amount of terrestrial sediment. These investigators also found high levels of fecal indicators in the run off from rain and nearby public showers. They also found feces of mongoose and pigeons, frequent
visitors, to contain high orders of magnitude of fecal indicators. The high levels found at Sans Souci have no obvious source, but may be influenced by bird droppings, soil, people, or other nearby surroundings. The incidence of high enterococci counts at sites influenced by terrestrial run-off during this study coincides with these earlier findings. Overall, there appeared to be little correlation between counts or recovery of \textit{S. aureus} with that of enterococci. However, there was a good relationship between total counts on CSA (gram positive bacteria) and enterococci (positive linear trend; \( R^2 = 0.95 \)). Other investigators have also found little correlation between counts of fecal indicators and the presence of pathogens in marine recreational waters (Papadakis et al., 1997; Yoshpe-Purer and Golderman, 1987; Solic and Krstulovic, 1994).

4. Non-anthropogenic sources of \textit{Staphylococcus aureus}

It has been reported that sand at marine recreational beaches can harbor pathogenic bacteria, including \textit{S. aureus} (WHO, 2004 and Papadakis et al., 1997). Concentrations of \textit{S. aureus} as high as 70 CFU/100 ml were recovered from beach sand samples in Greece (Papadakis et al., 1997). Prado et al. (1994) found that \textit{S. aureus} represented 24% of the gram-positive organisms recovered from beach sand at popular sites in Chile. Oshiro and Fujioka (1995) also recovered high levels of fecal indicator bacteria \((10^5 \text{ CFU/100 g})\) from sand around Oahu. In this present study, four sand samples collected from four sites were analyzed on CSA or CSA+A+G for \textit{S. aureus} to determine if beach sands on Oahu are a refuge and a possible source of this pathogen in recreational waters. Different areas of the beach were analyzed at each site. The “dry” area is that portion of the beach inland to the most recent tidal mark where the surface layer is visibly dry; majority of people are found on this area of beach. The “moist” area
lies between the dry area and the active surf zone where the surface layer is visibly still moist. The “swash” zone is the interface between land and sea where the surf is actively washing ashore and receding. *S. aureus* was recovered from two samples taken from two different sites out of nine samples and four sites. Both of these samples were from the “moist” area of the beach and each had a high level (>100 CFU/100 g) of *S. aureus* (Table 5.7). A significant level of Gram-positive bacteria (total counts on CSA) was recovered from the majority of the samples. The least amount of Gram-positive bacteria was recovered from the “swash” zone, evidence that there is a washing off of bacteria from sand in this area and possibly contaminating nearby water. This washing off effect was also suggested by Oshiro and Fujioka (1995) after their investigation in Hawaii.
Papadakis et al. (1997) also found greater levels of bacteria in sand than seawater. From the limited data collected in this study, there is an indication that sand can harbor *S. aureus* and may be a significant source for contamination of the nearby recreational waters.

*Staphylococcus aureus* has been found to have increased survivability in marine waters compared to fecal indicators (Gabutti et al., 2000; Solic and Krstulovic, 1994). Solic and Krstulovic (1994) found *S. aureus* populations to survive over 17 days in marine water in idealized conditions but this significantly decreased to only eight hours when exposed to several environmental stresses. Using several marine water samples collected from recreational beaches on Oahu, Charoenca (1991) found *S. aureus* survivability to vary somewhere between two to over three days with increased survival when a small amount of peptone was added. In the present study the survivability of *S. aureus* in marine waters was also explored by seeding cells into previously sterilized
seawater and sand collected from recreational beaches on Oahu. From the first seeding experiment it was found that *Staphylococcus aureus* cells survive at least 24 hours in seawater (approximately 50% decrease after 24 hours, Figure 5.3). In the next experiment, the addition of peptone facilitated increased survival and possibly growth of *S. aureus* in seawater. There were more colonies recovered from seawater with 0.0005% peptone than without peptone after 24 hours on both TSA (185 and 118 CFU/100ml respectively) and CSA (188 and 76 CFU/100ml respectively, Figures 5.4 and 5.5). However, there was not an increase between time zero and 24 hours in seawater with 0.0005% peptone (252 to 248 CFU/100ml on TSA and 266 to 252 CFU/100ml on CSA, Figures 5.4 and 5.5). No substantial decrease in cell numbers occurred in seawater with 0.0005% peptone until after 72 hours (206 to 44 CFU/100ml, Figure 5.4), suggesting that the peptone is providing the minimal amount of nutrients for cells to maintain their population size. A greater number of cells were nearly consistently recovered from seawater supplemented with 0.005% peptone than from other waters on both TSA and CSA (Figures 5.4 and 5.5). Even after five days of ambient incubation with exposure only to fluorescent lighting, the number of cells recovered from seawater with 0.005% peptone remained high (311 CFU/100ml, Figure 5.4). After 24 hours, both types of peptone supplemented seawaters exhibited an increase in cells recovered on TSA (185 to 236 and 306 CFU/100ml from 24 to 36 hours, Figure 5.4), which may reflect a lag time for *S. aureus* growth in seawater and/or an acclimation to the new environment.

When survivability on sand was tested, it was found that *Staphylococcus aureus* can grow (exponential curve) on sand with 10% seawater. The number of colonies recovered from sand that had been seeded with *S. aureus* increased up to about seven
times the initial number of cells recovered after about three days (270 to 1980 CFU/100 ml, Figure 5.6). It should be noted that the decline after 125 hours (Figure 5.6) may be due to the lack of moisture and thus dehydration of cells, as the moisture content was not corrected during this time where it had been earlier in the experiment.

The lack of nutrients in seawater is probably one of the limiting factors for the survivability of *S. aureus* in seawater. The *S. aureus* population declined in seawater without peptone but remained relatively stable when peptone was present, indicating that nutrients play a vital role in their survivability in seawater. Interestingly, *S. aureus* was not only stable on sand but was capable of exponential growth. Sand particles are not known to contain large amounts of nutrients though perhaps there are enough for *S. aureus* or perhaps some other factor such as biofilm formation is important. *S. aureus* has complex nutritional requirements and thus it is thought that this pathogen is not likely to multiply in most waters. From the present study, however, the only conclusion that can be construed is that *S. aureus* could survive in seawater in the presence of additional nutrients, though likely not increase in numbers, and can replicate when associated with sand particles. Additional research is needed to better assess the survivability of *S. aureus* in the environment. There are numerous environmental factors to consider when studying an organisms behavior in a natural environment that were not addressed in these experiments.

5. **Comparison of methods to recover *S. aureus* and MRSA from seawater**

The recovery of *S. aureus* was best seen on CSA however the increased inhibition of background, evident for both modified versions of CSA (CSA+A and CSA+A+G), proved useful in assessing highly contaminated waters. The recovery of *S. aureus* on
CSA without recovery on CSA+A+G occurred one-third of the time. Also, CSA+A recovered less than 50% of the *S. aureus* than on CSA from 32% of the samples and from 55.8% of the samples for CSA+A+G compared to CSA. As determined earlier by experimentation, the addition of glycine had a minimal affect on *S. aureus* recovery on CSA+A. Thus, the lower percentage of positive samples for CSA+A+G compared to CSA+A may be due to inherent differences in the samples, as no one sample was analyzed with both these modified media. Although recovery of *S. aureus* on CSA+A+G was poorer than CSA, this medium had increased inhibition of non-target cells that enabled correct identification of the target mauve colonies when assessing water samples with a relatively higher level of bacterial contamination. For 11.6% of the samples CSA+A+G recovered *S. aureus* when CSA failed, as well 16.3% of samples had higher *S. aureus* counts on CSA+A+G than CSA. Thus, CSA+A+G was useful as a back-up means to detect and recover greater quantities of *S. aureus*, particularly since increased inhibition of total cells allowed a larger volume of the samples to be analyzed. As mentioned previously, the ability to identify presumptive *S. aureus* colonies on CSA media is diminished when total colony counts in a sample exceeds 250 CFU/100ml (>125 colonies with only 50 ml of sample filtered). For 93.2% of the CSA plates, total colony counts exceeded this level but 76.7% of the CSA+A+G plates were below this level. Hence, identification of the mauve colored presumptive *S. aureus* colonies on CSA+A+G was eased by this medium’s ability to limit the total number of colonies recovered from seawater samples when compared to CSA. In conclusion, it is recommended that CSA+A+G be used in addition to CSA instead of replacing CSA, because the recovery
efficiency of CSA+A+G for *S. aureus* is poorer than CSA but is still useful in detecting *S. aureus* from highly contaminated waters or when larger volumes are analyzed.

Several methods to isolate MRSA from seawater samples were utilized in this study that resulted in the collection of 19 isolates (Table 5.8) which represented 5.7% of the isolates tested for resistance. One method in particular, screening for resistance amongst the confirmed *S. aureus* isolates recovered from CSA containing plates (CSA and CSA with either sodium pyruvate or polymyxin B), was particularly useful in the recovery of these MRSA. Approximately 5.7% of the isolates tested with this method were determined to be MRSA which represented 31.6% of the total MRSA isolated from seawater samples. Screening presumptive *S. aureus* isolates directly from primary isolation plates with CSA+O did not result in the recovery of any MRSA isolates, thus was not useful in selective recovery of MRSA from seawater. The direct plating of seawater samples onto CSA with oxacillin using membrane filtration resulted in the recovery of two MRSA isolates out of a total of 44 samples tested with this method. Different enrichment methods were also used to evaluate the presence of MRSA in nine samples and resulted in the isolation of six confirmed *S. aureus* isolates, four of these were determined to be oxacillin resistant (a.k.a. MRSA). Although the enrichment method was able to recover *S. aureus* isolates from seawater, all of the samples with *S. aureus* isolation from enrichment also had an *S. aureus* isolate with the same susceptibility to oxacillin recovered from membrane filtration methods. Thus, the enrichment methods employed in this study did not prove useful in enhancing the recovery of MRSA isolates from seawater samples. The screening of confirmed *S. aureus* isolated with CSA+O resulted in seven false positives, good growth (+), when
disk diffusion methods were used to confirm resistance. Some susceptible strains are capable of growth on CSA+O (Table 5.9). Indicating this method will not inhibit the recovery of MRSA strains since susceptible strains grew. Therefore, screening isolates for resistance after recovery from the primary isolation plate with CSA+O was the most useful method to isolate MRSA isolates from seawater samples. Perhaps the recovery was better with these methods because *S. aureus* cells are already stressed from being in seawater making direct recovery on a highly selective medium, such as CSA+O, difficult. Thus, when *S. aureus* is grown in a less stressful environment (e.g. laboratory incubation on general media) prior to plating onto CSA+O the recovery of resistant strains is improved. This theory is partly supported by the results from the Sans Souci sample taken during “Experiment 24” (Appendix D), in which a greater number of MRSA isolates were recovered on CSA+O after enrichment (enrich CSA+O) than directly from seawater (CSA+O). The isolate from membrane filtration (#24-37) was later determined to be the same strain type, by PFGE, as the only one enrichment isolate (#24-69) that was tested (Chapter 6, Appendix E). Standard methods for the recovery of stressed enteric bacterial cells from water samples using membrane filtration have been described (Greenberg et al., 1992). Some of these methods included pre-incubation of the membrane on a non-selective medium, usually a broth, prior to plating on a selective medium, the use of a two-layer agar in which a thin layer of general medium is poured over the selective medium, or deletion of the suppressive agent from the media. The basis of these methods are similar to what was found in this study, recovery of stressed cells can be enhanced if they are first placed in more ideal growth conditions prior to recovery.
on a selective medium. A two-layer agar technique was not evaluated in this study. This may be method of recovering stressed MRSA cells from seawater.

Although screening of previously recovered isolates appeared to improve recovery of MRSA strains, the use of this method to test every presumptive \textit{S. aureus} colony recovered on CSA is not feasible when levels of \textit{S. aureus} are high. Therefore, future studies are needed to improve methods for the selective recovery of MRSA from seawater. A new medium CHROMagar MRSA (cMRSA) was developed by the manufacturer after this study was conducted and may be more useful in direct membrane filtration methods for the recovery of MRSA from seawater. This medium is similar to CSA+O but employs the use of a cephalosporin antibiotic rather than oxacillin as the selective agent for MRSA. The use of cMRSA has been shown to be highly useful in detecting MRSA from clinical samples with a recovery 86\% of specimens evaluated by McKitrick and Halstead (2004) and a sensitivity of 96\% and specificity of 100\% reported by de Gialluly et al. (2004). Its applicability in the environment has yet to be explored and may not prove useful due to the greater number of non-multiresistant MRSA that are likely present.

6. \textbf{Summary of \textit{S. aureus} in marine waters of Oahu and recommendations}

The assessment of CSA and its modifications in the first phase of this study provided sufficient data for initial methods for the selection and identification of \textit{S. aureus}. This was necessary before a quantitative assessment of \textit{S. aureus} in marine recreational waters could be done. CSA gave the best recovery of \textit{S. aureus} from marine waters than the modified versions (CSA+A and CSA+A+G) but identification of target colonies was difficult when highly contaminated waters were analyzed with this medium.
Therefore, CSA+A and CSA+A+G were employed in conjunction with CSA in the subsequent analysis of beach waters. Additionally, several methods to improve recovery and select for MRSA strains were used in order to determine the prevalence of such strains in these waters. Monitoring of several marine recreational waters on Oahu was performed so that levels of *S. aureus* contamination at these sites could be determined. The primary findings of this study were that *S. aureus* counts correlate well with level of beach users without correlation to enterococci counts, that beach sand may serve as a reservoir of *S. aureus* and may contaminate nearby water and that *S. aureus* may survive prolonged periods seawater where there is an additional nutrient input. As a result of these studies, a total of 130 confirmed *S. aureus* have been placed in long-term storage for future analysis (Appendix D). The use of CSA+A+G in addition to CSA with membrane filtration methods is recommended to ensure an adequate assessment of *S. aureus* in marine waters. Finally, future studies are needed to improve methods for the selective recovery of MRSA from seawater.
Table 5.3. The concentrations of *Staphylococcus aureus* (CFU/100ml) and number of methicillin-resistant *S. aureus* (MRSA) or methicillin-intermediate *S. aureus* (MISA) isolates recovered from marine waters of Oahu with corresponding numbers of persons present (per approximately 250 m²) at time of sampling.

<table>
<thead>
<tr>
<th>Location</th>
<th># samples (# dates)</th>
<th>Data Type</th>
<th>Total People*</th>
<th># MRSA Isolates (# samples)</th>
<th># MISA Isolates (# samples)</th>
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<tr>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
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<tr>
<td></td>
<td>RANGE</td>
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<tr>
<td>Hanauma Bay</td>
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<td>1</td>
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<tr>
<td></td>
<td>RANGE</td>
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<tr>
<td>Duke</td>
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<td>GMN</td>
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<td>10</td>
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<tr>
<td>Kahanamoku</td>
<td></td>
<td>RANGE</td>
<td></td>
<td></td>
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</table>
Table 5.3 (Continued). The concentrations of *Staphylococcus aureus* (CFU/100ml) and number of methicillin-resistant *S. aureus* (MRSA) or methicillin-intermediate *S. aureus* (MISA) isolates recovered from marine waters of Oahu with corresponding numbers of persons present (per approximately 250 m²) at time of sampling.

<table>
<thead>
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<th>Location</th>
<th># samples (# dates)</th>
<th>Data Type</th>
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<th># MRSA Isolates (# samples)</th>
<th># MISA Isolates (# samples)</th>
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<td>7 (4)</td>
<td>2 (2)</td>
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<td>74</td>
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<tr>
<td>Makaha</td>
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<td>GMN</td>
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<td>RANGE</td>
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<tr>
<td>Haleiwa</td>
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<td>GMN</td>
<td>42</td>
<td>6</td>
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<td>1</td>
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</table>
Table 5.3 (Continued). The concentrations of *Staphylococcus aureus* (CFU/100ml) and number of methicillin-resistant *S. aureus* (MRSA) or methicillin-intermediate *S. aureus* (MISA) isolates recovered from marine waters of Oahu with corresponding numbers of persons present (per approximately 250 m²) at time of sampling.

<table>
<thead>
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<th></th>
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<th>Data Type</th>
<th>Total People*</th>
<th>S. aureus (# samples)</th>
<th># MRSA Isolates (# samples)</th>
<th># MISA Isolates (# samples)</th>
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<td></td>
<td></td>
<td>RANGE</td>
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<tr>
<td>Sand Island</td>
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<td>GMN</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RANGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waialae</td>
<td>1</td>
<td>GMN</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RANGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wailupe</td>
<td>1</td>
<td>GMN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RANGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala Wai</td>
<td>2 (2)</td>
<td>GMN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RANGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = average of total people present at the time of sampling; GMN = Geometric Mean
Table 5.4. *Staphylococcus aureus* (SA) and Gram-positive (Total) concentrations (CFU/100ml) recovered from marine waters on Oahu on several types of media.

<table>
<thead>
<tr>
<th>OVERALL</th>
<th>CSA (SA)</th>
<th>CSA (Total)</th>
<th>CSA+A (SA)</th>
<th>CSA+A (Total)</th>
<th>CSA+A+G (SA)</th>
<th>CSA+A+G (Total)</th>
<th>CSA+O (SA)</th>
<th>CSA+O (Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMN</td>
<td>7</td>
<td>919</td>
<td>19</td>
<td>306</td>
<td>1</td>
<td>52</td>
<td>0.3</td>
<td>83</td>
</tr>
<tr>
<td>AVG</td>
<td>22</td>
<td>1481</td>
<td>28</td>
<td>495</td>
<td>4</td>
<td>88</td>
<td>0.3</td>
<td>111</td>
</tr>
<tr>
<td>RANGE</td>
<td>0-124</td>
<td>15100</td>
<td>1-91</td>
<td>0-1420</td>
<td>0-42</td>
<td>6-TNTC</td>
<td>0</td>
<td>TNTC</td>
</tr>
<tr>
<td>SWIM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMN</td>
<td>9</td>
<td>878</td>
<td>19</td>
<td>306</td>
<td>1</td>
<td>56</td>
<td>0.3</td>
<td>74</td>
</tr>
<tr>
<td>AVG</td>
<td>24</td>
<td>1211</td>
<td>28</td>
<td>495</td>
<td>4</td>
<td>79</td>
<td>0.3</td>
<td>88</td>
</tr>
<tr>
<td>RANGE</td>
<td>0-124</td>
<td>&gt;4724</td>
<td>1-91</td>
<td>1513</td>
<td>0-42</td>
<td>6-TNTC</td>
<td>0</td>
<td>TNTC</td>
</tr>
<tr>
<td>NON-SWIM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMN</td>
<td>0</td>
<td>1696</td>
<td>N/A</td>
<td>N/A</td>
<td>1</td>
<td>36</td>
<td>0.3</td>
<td>144</td>
</tr>
<tr>
<td>AVG</td>
<td>1</td>
<td>5156</td>
<td>N/A</td>
<td>N/A</td>
<td>2</td>
<td>143</td>
<td>0.3</td>
<td>232</td>
</tr>
<tr>
<td>RANGE</td>
<td>0-2</td>
<td>15100</td>
<td>N/A</td>
<td>N/A</td>
<td>0-9</td>
<td>6-TNTC</td>
<td>0</td>
<td>TNTC</td>
</tr>
</tbody>
</table>

CSA = CHROMagar™ Staph aureus, CSA+A = CSA with sodium azide, CSA+A+G = CSA+A with glycine, CSA+O = CSA with oxacillin; GMN = geometric mean, AVG = arithmetic mean/average; Swimming = sites with consistently >10 people present at time of sampling; Non-swimming = sites with consistently <10 people present at time of sampling; N/A = not applicable, TNTC = too numerous to count.
Table 5.5. Concentrations of *Staphylococcus aureus* (CFU/100 ml) recovered from samples of seawater by season (summer versus winter), site (Kuhio, Sans Souci, and Ala Moana), and degree of use (swimming versus non-swimming).

<table>
<thead>
<tr>
<th></th>
<th>Summer (May-October)</th>
<th>Winter (November-April)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero</td>
<td>Geometric Mean</td>
</tr>
<tr>
<td>Site</td>
<td>n (%)</td>
<td>All days</td>
</tr>
<tr>
<td>Kuhio</td>
<td>1 (8.3)</td>
<td>14 (n=12)</td>
</tr>
<tr>
<td>Sans Souci</td>
<td>0 (0)</td>
<td>40 (n=11)</td>
</tr>
<tr>
<td>Ala Moana</td>
<td>0 (0)</td>
<td>10 (n=12)</td>
</tr>
<tr>
<td>Swimming</td>
<td>2 (3.6)</td>
<td>11 (n=55)</td>
</tr>
<tr>
<td>Non-swimming</td>
<td>1 (50)</td>
<td>0 (n=1)</td>
</tr>
</tbody>
</table>

Zero = *S. aureus* not recovered; Swimming = sites with consistently >10 people present at time of sampling; Non-swimming = sites with consistently <10 people present at time of sampling.
Table 5.6. Levels of enterococci, *Staphylococcus aureus*, and Gram-positive bacteria (CFU/100ml) recovered from marine waters of Oahu.

<table>
<thead>
<tr>
<th>D.O.C.</th>
<th>Location</th>
<th>Enterococci</th>
<th><em>S. aureus</em></th>
<th>Gram-Positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/29/04</td>
<td>Kuhio</td>
<td>0</td>
<td>2</td>
<td>1120</td>
</tr>
<tr>
<td></td>
<td>Sand Island</td>
<td>4</td>
<td>0</td>
<td>3620</td>
</tr>
<tr>
<td></td>
<td>Wailupe</td>
<td>2280</td>
<td>0</td>
<td>15100</td>
</tr>
<tr>
<td>3/14/04</td>
<td>Sans Souci</td>
<td>8</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Ala Wai**</td>
<td>674</td>
<td>0</td>
<td>6000</td>
</tr>
<tr>
<td>6/11/04</td>
<td>Kuhio</td>
<td>2</td>
<td>0</td>
<td>282</td>
</tr>
<tr>
<td></td>
<td>Kailua</td>
<td>12</td>
<td>2</td>
<td>398</td>
</tr>
<tr>
<td></td>
<td>Ala Wai**</td>
<td>0</td>
<td>41</td>
<td>1330</td>
</tr>
<tr>
<td>7/17/04</td>
<td>Kuhio</td>
<td>10</td>
<td>2</td>
<td>2010</td>
</tr>
<tr>
<td></td>
<td>Hanauma Bay</td>
<td>7</td>
<td>20</td>
<td>990</td>
</tr>
</tbody>
</table>

* = Total colony counts on CHROMagar™ Staph aureus; ** = Brackish water canal; D.O.C. = date of collection

Table 5.7. Concentrations (CFU/100g O.D. sand) of *Staphylococcus aureus* and Gram-positive bacteria recovered from beach sands that were sampled from different areas (dry, moist, swash) of popular beaches on Oahu.

<table>
<thead>
<tr>
<th>Sampling Site</th>
<th>Dry</th>
<th>Moist</th>
<th>Swash</th>
<th>Dry</th>
<th>Moist</th>
<th>Swash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala Moana</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.2x10⁴</td>
<td>1.2x10⁴</td>
<td>0</td>
</tr>
<tr>
<td>Kuhio</td>
<td>0</td>
<td>2.6x10²</td>
<td>n.d.</td>
<td>2.9x10⁵</td>
<td>7.8x10⁴</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sans Souci</td>
<td>0</td>
<td>0</td>
<td>n.d.</td>
<td>1.3x10⁴</td>
<td>1.0x10⁴</td>
<td>n.d.</td>
</tr>
<tr>
<td>Kailua</td>
<td>n.d.</td>
<td>1.4x10²</td>
<td>0</td>
<td>TNTC</td>
<td>1.4x10³</td>
<td>8.1x10⁴</td>
</tr>
</tbody>
</table>

n.d. = no data

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Table 5.8. Number of methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-intermediate S. aureus (MISA) recovered using different media and number of samples tested with each.

<table>
<thead>
<tr>
<th>No. Samples tested</th>
<th>MRSA</th>
<th>MISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total = 19</td>
<td>Total = 13</td>
</tr>
<tr>
<td>76</td>
<td>CSA = 6</td>
<td>CSA = 10</td>
</tr>
<tr>
<td>25</td>
<td>CSA+A = 2</td>
<td>CSA+A+G = 2</td>
</tr>
<tr>
<td>42</td>
<td>CSA+A+G = 1</td>
<td>CSA+A = 1</td>
</tr>
<tr>
<td>8</td>
<td>C+2O = 1</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>CSA+O = 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E = 4*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CSA+X = 4</td>
<td></td>
</tr>
</tbody>
</table>

* = All four isolates recovered from the same enrichment; CSA = CHROMagar™ Staph aureus, CSA+A = CSA with 0.0035% sodium azide, CSA+A+G = CSA+A with 12g/l glycine, C+2O = CSA with 2µg/ml oxacillin, CSA+O = CSA with 4µg/ml oxacillin, E = enrichment, C+X includes CSA with sodium pyruvate or Polymyxin B

Table 5.9. Results from the testing of confirmed Staphylococcus aureus isolates for oxacillin resistance by different methods (n=332; 63 tested with both methods).

<table>
<thead>
<tr>
<th></th>
<th>Screen Plate</th>
<th>Disk Diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL</td>
<td>141</td>
<td>254</td>
</tr>
<tr>
<td>MSSA</td>
<td>78</td>
<td>222</td>
</tr>
<tr>
<td>MISA</td>
<td>38</td>
<td>13</td>
</tr>
<tr>
<td>MRSA</td>
<td>25</td>
<td>19</td>
</tr>
</tbody>
</table>

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Figure 5.3. Time series of initial growth experiment for *Staphylococcus aureus* recovered from seeded filter-sterilized seawater samples using tryptic soy agar (TSA) and CHROMagar™ Staph aureus (CSA).

Figure 5.4. Time series of *Staphylococcus aureus* recovered from unseeded filter-sterilized seawater (control), seeded filter-sterilized seawater (NP), seeded filter-sterilized seawater with 0.0005% peptone (0.0005), and seeded filter-sterilized seawater with 0.005% peptone (0.005) using tryptic soy agar.
**Figure 5.5.** Time series of *Staphylococcus aureus* recovered from unseeded filter-sterilized seawater (control), seeded filter-sterilized seawater (NP), seeded filter-sterilized seawater with 0.0005% peptone (0.0005), and seeded filter-sterilized seawater with 0.005% peptone (0.005) using CHROMagar™ Staph aureus.

**Figure 5.6.** Time series of *Staphylococcus aureus* recovered from seeded sand sample on CSA minus colonies recovered from control sand sample on tryptic soy agar (NOTE: moisture content not adjusted after 125 hours; TNTC = too numerous to count).
CHAPTER SIX
Typing of Isolates Recovered from Marine Waters with Comparison to Clinical Isolates

A. Objectives

The primary objective for this final phase of the study was to characterize the Staphylococcus aureus isolates recovered from marine recreational waters and compare them with those recovered from clinical samples to determine if recreational waters are a possible source for their transmission. These isolates were characterized based on antibiotic resistance profiles, ribotypes, genotypic fingerprints and/or pattern of virulence genes present.

B. Materials and Methods

1. Staphylococcus aureus isolates

Environmental S. aureus are those isolates that were recovered from marine waters during this study (Appendices D, E, and F). Clinical S. aureus are isolates that were acquired from a local diagnostic laboratory, which processes clinical samples from clinics and hospitals throughout the state of Hawaii.

2. Multiple antibiotic susceptibility testing

In order to establish antibiotic susceptibility profiles of the confirmed S. aureus strains recovered from marine waters, the disk diffusion method (as described for oxacillin testing) was used to test these isolates against several antibiotics (Table 6.1). At least one representative from each major class of antibiotics was chosen. As indicated in the introduction, susceptibility tetracycline is reported to be useful in discerning
community acquired strains of MRSA. Minimum inhibitory concentrations to oxacillin were also determined using E-test (ABbiodisk).

3. RiboPrinter® from DuPont Qualicon

Ribotyping of isolates has been shown to be useful in grouping related strains (Kishimoto et al, 2004). This process can be tedious and expensive but a new automated system has made this analysis much more efficient. This system entails preparing a sample of fresh culture in buffer, heat treating, and then loading into the automated machine. Within the machine the DNA is prepped (isolated and cut with enzyme, in this case EcoR1) separated (electrophoresis) and transferred (membrane), membrane of banding pattern processed, image of membrane pattern taken and then recorded image logged into the database where the resulting Riboprint® can be compared to the manufacturer’s database and to operators previous samples.

4. Molecular typing analyses performed at external laboratories.

Molecular typing of S. aureus is commonly performed by Pulsed-Field Gel Electrophoresis (PFGE). This is performed by preparing deoxyribonucleic acid (DNA) plugs (DNA suspended in small sections of agarose gel) from fresh cultures grown in brain heart infusion broth, and using a slice from the plug to cut the DNA using the digestive enzyme SmaI. Then, the resulting fragments are passed through an agar gel using pulsed-field electrophoresis. Finally, the resulting banding patterns are stained and documented. Pulsed-field gel changes the orientation of the anode and cathode during the electrophoresis process and the preparation of the plug provides added stability to the whole genome, these qualities allow larger fragments of DNA to be analyzed than what can be done using standard electrophoresis procedures. All analyses were performed in
collaboration with Hawaii State Department of Health laboratory personnel (Dana Tamashiro) following PulseNet\textsuperscript{TM} procedures. Many of the same isolates analyzed at the state labs were also analyzed with the same procedures at the Center for Disease Control (CDC) in Atlanta, Georgia.

The staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}), discussed in Chapter one, carries the genes that encode PBP2\textsuperscript{'}, the protein that confers resistance to methicillin and its derivatives. Four types of SCC\textit{mec} have been identified with type IV being primarily associated with CA-MRSA. Typing of SCC\textit{mec} was done for several confirmed \textit{S. aureus} isolates (n=31) recovered from seawater samples at the CDC in Atlanta, Georgia using multiplex PCR techniques.

The virulence of different strains of \textit{S. aureus} can be inferred by the presence of particular genes and the pattern of genes present can be used to distinguish between strains. Typically PCR techniques are used to detect the presence of selected known virulence genes in the genome of a particular isolate. The 31 isolates sent to the CDC were analyzed by PCR methods for the presence of eight toxin genes (Table 6.2).
<table>
<thead>
<tr>
<th>Specific Substance</th>
<th>Antimicrobial Group</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin disks (5mcg)</td>
<td>Glycopeptide</td>
<td>Inhibits cross-linkage of peptidoglycan layers</td>
</tr>
<tr>
<td>Tetracycline disks (30 mcg)</td>
<td>Tetracycline</td>
<td>Prevents polypeptide elongation at 30S ribosome</td>
</tr>
<tr>
<td>Gentamicin disks (10mcg)</td>
<td>Aminoglycoside</td>
<td>Generates early release of abnormal peptide chains from 30S ribosome</td>
</tr>
<tr>
<td>Penicillin disks (10 U)</td>
<td>β-lactam – penicillin</td>
<td>Binds PBPs and enzymes responsible for peptidoglycan synthesis</td>
</tr>
<tr>
<td>Ampicillin disks (10mcg)</td>
<td>β-lactam – penicillin</td>
<td>Binds PBPs and enzymes responsible for peptidoglycan synthesis</td>
</tr>
<tr>
<td>Erythromycin disks (15mcg)</td>
<td>Macrolide</td>
<td>Prevents polypeptide elongation at 50S ribosome</td>
</tr>
<tr>
<td>Cefotaxime disks (30mcg)</td>
<td>β-lactam – cephalosporin</td>
<td>Binds PBPs and enzymes responsible for peptidoglycan synthesis</td>
</tr>
<tr>
<td>Ciprofloxacin disks (5mcg)</td>
<td>Quinolone</td>
<td>Binds α subunit of DNA gyrase</td>
</tr>
</tbody>
</table>
Table 6.2. The virulence genes that were analyzed at Center for Disease Control using PCR methods to detect their presence within confirmed *Staphylococcus aureus* isolates.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Virulence factor</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mecA</em></td>
<td>Methicillin resistance determinant</td>
</tr>
<tr>
<td><em>tss</em></td>
<td>Toxic shock syndrome toxin</td>
</tr>
<tr>
<td><em>sea</em></td>
<td>Enterotoxin A</td>
</tr>
<tr>
<td><em>seb</em></td>
<td>Enterotoxin B</td>
</tr>
<tr>
<td><em>sec</em></td>
<td>Enterotoxin C</td>
</tr>
<tr>
<td><em>sed</em></td>
<td>Enterotoxin D</td>
</tr>
<tr>
<td><em>see</em></td>
<td>Enterotoxin E</td>
</tr>
<tr>
<td><em>seh</em></td>
<td>Enterotoxin H</td>
</tr>
<tr>
<td>PVL</td>
<td>Panton-Valentine Leukocidin</td>
</tr>
</tbody>
</table>

* SCC*mec* typing
C. Results and Discussion

1. Antibiotic sensitivity patterns of *S. aureus* isolates recovered from seawater

The antibiotic sensitivities to eight antibiotics (Table 6.3) were performed on 48 isolates of *S. aureus* recovered from marine waters (11 MRSA) and 50 isolates from clinical cultures (18 MRSA). Most of the *S. aureus* isolates recovered from both sources were resistant to penicillin and ampicillin. The percent resistance to these two antibiotics for environmentally derived *S. aureus* isolates was 93.8%, whereas the percentage for clinical isolates was lower at 90.0%. In contrast, most of the clinical and environmental MSSA isolates remained sensitive to the last six antibiotics listed in Table 6.3. The range in percentage of *S. aureus* isolates sensitive to these antibiotics was from 75.0% to 100% for environmentally derived isolate and from 64.0% to 100% for clinically derived isolates. The majority of clinical MRSA isolates were resistant to all β-lactams and erythromycin (83.3%), this pattern was also seen in three environmental isolates. The majority of the MSSA strains from both clinical and environmental sources were resistant to both penicillin and ampicillin. Based on different patterns of susceptibility, the *S. aureus* isolates from the environment were more diverse than clinical isolates (Table 6.4). Amongst the *S. aureus* isolates from environment and clinical specimens, the MRSA isolates within these groups exhibited greater diversity than less resistant isolates. In their study conducted at a hospital in Honolulu, Hawaii, Fishbain et al. (2003) found that 28% of inpatient derived MRSA isolates and 62% of outpatient isolates were sensitive to erythromycin. Their results differ from those of this study in which 10% of the inpatient MRSA isolates and 25% of the outpatient isolates were sensitive to erythromycin. A
greater number of the environmental isolates, however, were susceptible to this antibiotic (45%). This is one indicator that MRSA in the environment are more like outpatient MRSA or CA-MRSA. Tetracycline is the only antibiotic used in this study that is considered a key antibiotic to distinguish CA-MRSA isolates, in which CA-MRSA are typically sensitive. In this study it was found that the majority of all of the *S. aureus* tested were tetracycline sensitive with only one clinical MSSA, five environmental MRSA and two environmental MSSA that were resistant to tetracycline. Therefore, in this study tetracycline was not useful as an indicator of community over hospital association amongst MRSA. Other studies involving susceptibility patterns of *S. aureus* isolates have found that generally a greater percentage of hospital associated or multi-resistant MRSA strains are resistant to erythromycin, ciprofloxacin, tetracycline and gentamicin (Gosbell et al., 2001; Okuma et al., 2002; Charlebois et al., 2002). If environmental MRSA isolates are considered to be primarily community associated or non-multiresistant, then the results of this study are generally in agreement with these other investigators’ findings. One exception is that an environmental isolate was resistant to gentamicin whereas all of the clinical isolates were susceptible. The overall antibiotic sensitivity results show that environmental *S. aureus* strains recovered from seawater are more diverse than hospital strains, that the majority of the environmental MRSA were likely community associated, non-multiresistant strains, and that there is some similarity between susceptibility patterns of isolates from environmental and clinical sources.

2. **Genotypic characterization based on ribosomal and chromosomal analyses**

Genotyping techniques further characterize strains of bacteria after initial identification. These are essentially “fingerprinting” techniques that establish individual
differences amongst strains, which is useful in determining common sources of bacteria particularly in outbreak studies.

Ribotyping is an example of such a technique that involves the enzymatic digesting of ribosomal genes with subsequent separation of the resulting fragments by size, which forms a distinctive "banding" pattern or fingerprint. By comparing the similarities between the different banding patterns amongst strains, they can be grouped based on the percentage of similarity they share. It is assumed that a particular strain will have a certain banding pattern and other strains that are closely related to that strain will share a very similar pattern, thus have a high percentage of similarity. The DuPont Riboprinter™, a fully automated system, performs such an analysis with the grouping of strains tested completed internally using preset parameters. This system also compares banding patterns of the tested strains to the patterns present in their database. Thus, a DuPont ID (comparison with database) is given as well as a ribogroup (comparison amongst tested isolates). A total of 73 *S. aureus* isolates were analyzed by the Riboprinter™ using an ecoR1 digest (48 MSSA, 9 MISA and 16 MRSA). Overall there were 47 different ribogroups with the majority of these isolates being the sole members of the group, which suggests there is considerable diversity amongst these isolates. However, there were three groups with four or more members, primarily MSSA. The MRSA isolates clustered into ten groups, in which three of these groups had three different MRSA isolates in each. The most common ribogroup (STAPH 277-89-S-4) included seven isolates and all of these also shared the same DuPont ID (DUP-14190). There was often a discrepancy between the DuPont group and assigned ribogroup amongst the isolates tested. This is because the ribogroup is formed on a sliding scale,
grouping isolates based on how their banding patterns cluster within a dendrogram comparison amongst those tested, and the DuPont ID is the closest matching pattern between the test isolate and isolates already in the database. When the ribotypes of these environmental isolates (n=72) were compared to those of clinical isolates (n=50), three major groups were still apparent. Two of these major groups are comprised of the same environmental isolates as the groups mentioned previously, with a few new clinical isolates. One of these major groups, comprised primarily of clinical isolates, was newly expanded from a single member group. Again, the most common ribogroup that included both environmentally and clinically derived S. aureus isolates was STAPH 277-89-S-4 with the addition of four clinical isolates to the seven environmental isolates already mentioned. Three of these clinical isolates also shared the same DuPont ID (DUP-14190) as the environmental S. aureus isolates. However, the most common DuPont ID (DUP-4001) included eight clinical and three environmental S. aureus isolates. Although the origin for most of the isolates used to form DuPont database are unknown, DUP-4001 is an ATCC reference strain (#14458) which was isolated from the feces of a child with diarrhea. Of the 50 clinical isolates tested, seventeen isolates formed nine groups that included environmental isolates while 15 of these isolates also shared the same DuPont ID as an environmental S. aureus isolate. Overall, the clinical isolates had 34 groups and a total of 64 groups were formed when these were paired with the environmental groups. This grouping of several environmental and clinical isolates by ribosomal patterning reveals that similar S. aureus strains can be recovered from these seemingly discrete settings, marine recreational waters and clinical specimens.
Another form of genotyping that is commonly used to characterize *S. aureus* is the PFGE method. This is also an enzymatic digest but differs from ribotyping in that it is a digest of the entire genomic DNA. Similar to ribotyping, the fragments that are a result of this digest are separated based on size into a “fingerprint” for comparison. This method may provide a better distinction amongst strains as it uses the entire genome instead of just the ribosomal related genes. Center for Disease Control (CDC) approved this method for use in investigations of outbreaks uses *SmaI* to cut the genome. The nucleotide pattern that *SmaI* targets typically is rare within the genome, which reduces the number of resulting bands. Using this procedure, CDC has developed an extensive database to compare the various strains of *S. aureus* throughout the country. The Hawaii Department of Health (DOH) and CDC performed the analysis in this study separately. The banding patterns generated from DOH analysis (Figure 6.1) were compared with a database in Washington (Washington State Public Health Laboratories, Ravi Pallipamu), while the patterns generated by the CDC were compared to their national database. McDougal et al. (2003) reported a previous classification of infection outbreak strains based on 80% similarity. These classifications have groups that typically contain isolates from community or hospital associated outbreaks, thus providing some distinction for the origin of our marine water isolates. The classification presented by Washington and CDC correlate well, the only difference was that Washington failed to classify some isolates that CDC could group. This was likely a reflection of the difference in the size of the databases used, where CDC’s is much more extensive. A dendrogram of the PFGE banding patterns generated from the DOH lab reveals that isolates from the same sample were either identical or highly similar and that there was more diversity amongst the
MSSA isolates than the MRSA (Figure 6.1 and Table 6.5). When the PFGE patterns generated from CDC were compared to the national database, 18 isolates matched with 8 different previously established PFGE types (PFT). One of the most common PFTs found in this study, USA100, is typically related to health care associated isolates that are resistant to erythromycin. Most of the isolates in this study with a PFT of USA100 were recovered from the same sample and were found to be erythromycin resistant with a SCCmec II; indicating they are likely HA-MRSA strains. Three other MRSA isolates analyzed by CDC matched with PFTs that are usually linked with community associated isolates (USA300, USA1000, and USA1100). Typically USA300 isolates carry SCCmec IV, are resistant to β-lactams, and are frequently from community onset skin infections. The isolate in this study that matched with USA300 (#8-82), carried SCCmec IV and was resistant to the β-lactams tested. The USA1000 and USA1100 types are newer groups than those designated in the report by McDougal et al. (2003), the isolates found in these groups typically carry the PVL gene and USA1000 isolates frequently carry the seb gene as well (Tenover, 2004). In this study, the isolate that matched with USA1100 (#17-76) was positive for the PVL gene whereas the USA1000 (#10-1) isolate did not carry the PVL gene but did carry the seb gene. PFGE analysis proved very useful in distinguishing amongst the S. aureus isolates recovered from seawater samples. Since the majority of these isolates had a nationally recognized PFT, there appears to be a number of clinically important S. aureus strains that are recoverable from popular marine recreational waters on Oahu.

As mentioned in the "Introduction", SCCmec carries the mecA gene which encodes PBP2'. This cassette has been found to occur in many forms, from a "primitive" form
encoding for resistance to β-lactams to an "advanced" form that includes resistance to multiple antibiotics. The typing of this cassette has been important in assessing the origin of MRSA outbreak strains as multiple antibiotic resistant is more typical of hospital outbreaks over community outbreaks. Thirty-one S. aureus isolates recovered from seawater samples were analyzed by lab personnel at CDC to determine their SCCmec type. All of the isolates determined to be MRSA using the methods of this study that were analyzed for SCCmec (n = 12) had this chromosomal cassette, indicating that the methods used to determine resistance in this study were reasonably accurate. Seven of these isolates, recovered from four separate seawater samples, had a SCCmec IV, two were IV-A and four, all from the same sample, were IV-C. The remaining five isolates, also recovered from four different seawater samples, had SCCmec II, one of these had an additional type IV-B band. Also not designated to a common PFGE group was the isolate with a IV-B band (17-39). Besides resistance to the penicillins, this isolate acquired resistance to erythromycin. This indicates that the 17-39 MRSA isolate may have recently acquired resistance to additional antibiotics. All of the SCCmec II were designated as PFGE type USA100 and exhibited resistance to one or more of the non-β-lactam antibiotics tested (Table 6.3). Overall, these results indicate that SCCmec types which are common to both CA-MRSA and HA-MRSA are present in seawater with a nearly equal incidence.

As mentioned, S. aureus strains can carry several virulence factors most marked by their array of toxin production. The toxin genes a particular strain harbors is therefore of interest as it provides evidence for its potential as a pathogen and further characterizes the strain. Toxin gene analysis is performed using PCR techniques. Thirty-one isolates were
tested using these techniques by CDC personnel. The genes analyzed included those listed in Table 2 of “Materials and Methods” section. Of the isolates tested, enterotoxin genes were found in 16, Tsst-1 in nine, and PVL in another two. There were nine different patterns of toxin genes detected amongst these strains, none of the patterns consistently matched with PFGE or SCCmec types unless they were isolates that were recovered from the same seawater sample. Thus, toxin gene patterns were not useful in distinguishing between strains, rather this data provides important information on the potential virulence of *S. aureus* strains found in seawater. The presence of the PVL gene was found in two of the MRSA isolates which had SCCmec IV-A, PFGE types USA 300 and USA1100, and were resistant only to β-lactam antibiotics. All of these traits, including the presence of PVL gene, have been reported to be indicative of CA-MRSA strains and suggested to confer a selective advantage for these pathogens (McDougal et al., 2003; Okuma et al., 2003). The Panton-Valentine leukocidin toxin that is encoded by the PVL gene is strongly associated with increased severity of skin infections. Thus, the presence of this gene in seawater MRSA isolates indicates the potential to cause severe skin infections amongst those exposed to contaminated seawater. The presence of TSST-1 gene within seawater *S. aureus* isolates is also a concern. This gene encodes a superantigen that is responsible for a severe and sometimes systemic disease of cutaneous and soft tissue, toxic shock syndrome. Interestingly, the DUP-4001 isolate is known to carry the enterotoxin B gene, but the results for the two isolates from this study that closely matched the DUP-4001 riboprint which were tested for the presence of this gene were negative. These results from toxin gene analysis of seawater *S. aureus* isolates
provide evidence that the majority of these isolates carry genes that encode known
virulence factors, with some having the genes for toxins that can confer severe infections.

3. Summary and conclusions

In order to determine if the presence of *S. aureus* and MRSA in marine recreational
waters poses a public health threat, it is important to show that some connection between
these environmentally derived isolates and those present in clinical specimens. In an
attempt to address this, several *S. aureus* and MRSA isolates recovered from marine
recreational water samples were typed using phenotypic and genotypic methods and then
these results compared with those from clinical specimens. The overall results from these
various analyses suggests that the environmental *S. aureus* strains, especially MSSA
strains, were more diverse than hospital strains, that both CA-MRSA and HA-MRSA are
present in seawater, that the majority of these isolates carry genes that encode known
virulence factors, and that several of these environmental isolates were similar to those
derived from clinical sources. Therefore, it appears that similar *S. aureus* strains can be
recovered from these seemingly discrete settings, marine recreational waters and clinical
specimens. This suggests that seawater may serve as a source for transmission of virulent
strains of *S. aureus* and MRSA, which has important public health implications.
Table 6.3. Percentage distribution of antibiotic sensitivities of *Staphylococcus aureus* recovered from environmental waters and from clinical specimens.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Culture Source</th>
<th>Resistant (%)</th>
<th>Intermediate (%)</th>
<th>Sensitive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>Environmental</td>
<td>35.4</td>
<td>12.5</td>
<td>52.1</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>36.0</td>
<td>0</td>
<td>64.0</td>
</tr>
<tr>
<td>Penicillin</td>
<td>Environmental</td>
<td>93.8</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>90.0</td>
<td>0</td>
<td>10.0</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Environmental</td>
<td>93.8</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>90.0</td>
<td>0</td>
<td>10.0</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>Environmental</td>
<td>20.8</td>
<td>4.2</td>
<td>75.0</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>34.0</td>
<td>0</td>
<td>66.0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Environmental</td>
<td>2.1</td>
<td>0</td>
<td>97.9</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Environmental</td>
<td>12.5</td>
<td>4.2</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>2.0</td>
<td>0</td>
<td>98.0</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Environmental</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Environmental</td>
<td>14.6</td>
<td>8.3</td>
<td>77.1</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>36.0</td>
<td>0</td>
<td>64.0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Environmental</td>
<td>14.6</td>
<td>10.4</td>
<td>75.0</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>18.0</td>
<td>0</td>
<td>82.0</td>
</tr>
</tbody>
</table>
Table 6.4. Comparative diversity of *Staphylococcus aureus* recovered from environmental waters and clinical specimens based on pattern of antibiotic susceptibility.

<table>
<thead>
<tr>
<th></th>
<th>Environmental (no. Groups/ no. Isolates)</th>
<th>Clinical (no. Groups/ no. Isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA</td>
<td>13/17 (77%)</td>
<td>3/17 (18%)</td>
</tr>
<tr>
<td>MSSA</td>
<td>6/25 (24%)</td>
<td>5/33 (15%)</td>
</tr>
<tr>
<td>MISA</td>
<td>4/6 (7%)</td>
<td>None</td>
</tr>
</tbody>
</table>

MRSA = methicillin-resistant *S. aureus*, MSSA = methicillin-susceptible *S. aureus*, MISA = methicillin-intermediately resistant *S. aureus*

Table 6.5. Characteristics of isolates analyzed by PFGE at Hawaii Department of Health.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Type</th>
<th>D.O.C.</th>
<th>Location</th>
<th>Oxacillin</th>
<th>LabID</th>
</tr>
</thead>
<tbody>
<tr>
<td>N04-136</td>
<td>Environmental</td>
<td>11/1/03</td>
<td>Ala Moana</td>
<td>Resistant</td>
<td>23-02</td>
</tr>
<tr>
<td>N04-137</td>
<td>Environmental</td>
<td>10/18/03</td>
<td>Sans Souci</td>
<td>Sensitive</td>
<td>20-26</td>
</tr>
<tr>
<td>N04-138</td>
<td>Environmental</td>
<td>11/1/03</td>
<td>Ala Moana</td>
<td>Resistant</td>
<td>23-44</td>
</tr>
<tr>
<td>N04-139</td>
<td>Environmental</td>
<td>11/1/03</td>
<td>Ala Moana</td>
<td>Resistant</td>
<td>23-50</td>
</tr>
<tr>
<td>N04-140</td>
<td>Environmental</td>
<td>9/6/03</td>
<td>Sans Souci</td>
<td>Sensitive</td>
<td>14-61</td>
</tr>
<tr>
<td>N04-141</td>
<td>Environmental</td>
<td>8/16/03</td>
<td>Kuhio</td>
<td>Sensitive</td>
<td>12-F</td>
</tr>
<tr>
<td>N04-142</td>
<td>Environmental</td>
<td>9/6/03</td>
<td>Sans Souci</td>
<td>Sensitive</td>
<td>14-64</td>
</tr>
<tr>
<td>N04-143</td>
<td>Environmental</td>
<td>9/20/03</td>
<td>Kuhio</td>
<td>Sensitive</td>
<td>17-19</td>
</tr>
<tr>
<td>N04-144</td>
<td>Environmental</td>
<td>8/16/03</td>
<td>Sans Souci</td>
<td>Sensitive</td>
<td>12-55</td>
</tr>
<tr>
<td>N04-145</td>
<td>Environmental</td>
<td>8/16/03</td>
<td>Kuhio</td>
<td>Sensitive</td>
<td>12-52</td>
</tr>
<tr>
<td>N04-146</td>
<td>Environmental</td>
<td>7/20/03</td>
<td>Magic Island</td>
<td>Sensitive</td>
<td>8-02</td>
</tr>
<tr>
<td>N04-148</td>
<td>Environmental</td>
<td>11/22/03</td>
<td>Sans Souci</td>
<td>Resistant</td>
<td>24-37</td>
</tr>
<tr>
<td>N04-149</td>
<td>Environmental</td>
<td>11/22/03</td>
<td>Kuhio</td>
<td>Sensitive</td>
<td>24-09</td>
</tr>
<tr>
<td>N04-150</td>
<td>Environmental</td>
<td>11/1/03</td>
<td>Ala Moana</td>
<td>Resistant</td>
<td>23-51</td>
</tr>
<tr>
<td>N04-151</td>
<td>Environmental</td>
<td>11/22/03</td>
<td>Kuhio</td>
<td>Sensitive</td>
<td>24-07</td>
</tr>
<tr>
<td>N04-152</td>
<td>Environmental</td>
<td>8/3/03</td>
<td>Sans Souci</td>
<td>Resistant</td>
<td>10-01</td>
</tr>
<tr>
<td>N04-153</td>
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<td>8/3/03</td>
<td>Magic Island</td>
<td>Sensitive</td>
<td>10-16</td>
</tr>
<tr>
<td>N04-154</td>
<td>Environmental</td>
<td>8/3/03</td>
<td>Sans Souci</td>
<td>Sensitive</td>
<td>10-21</td>
</tr>
<tr>
<td>N04-155</td>
<td>Environmental</td>
<td>7/20/03</td>
<td>Sans Souci</td>
<td>Sensitive</td>
<td>8-123</td>
</tr>
<tr>
<td>N04-156</td>
<td>Environmental</td>
<td>10/4/03</td>
<td>Ala Moana</td>
<td>Sensitive</td>
<td>18-01</td>
</tr>
<tr>
<td>N04-157</td>
<td>Environmental</td>
<td>10/4/03</td>
<td>Kuhio</td>
<td>Sensitive</td>
<td>18-12</td>
</tr>
<tr>
<td>N04-158</td>
<td>Environmental</td>
<td>10/4/03</td>
<td>Sans Souci</td>
<td>Sensitive</td>
<td>18-30</td>
</tr>
<tr>
<td>N04-159</td>
<td>Environmental</td>
<td>10/4/03</td>
<td>Kuhio</td>
<td>Sensitive</td>
<td>18-65</td>
</tr>
<tr>
<td>N04-160</td>
<td>Environmental</td>
<td>9/20/03</td>
<td>Ala Moana</td>
<td>Resistant</td>
<td>17-76</td>
</tr>
<tr>
<td>N04-161</td>
<td>Environmental</td>
<td>9/20/03</td>
<td>Sans Souci</td>
<td>Resistant</td>
<td>17-39</td>
</tr>
<tr>
<td>N04-162</td>
<td>Environmental</td>
<td>11/22/03</td>
<td>Sans Souci</td>
<td>Resistant</td>
<td>24-69</td>
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<tr>
<td>N04-163</td>
<td>Environmental</td>
<td>1/17/04</td>
<td>Kuhio</td>
<td>Resistant</td>
<td>28-37</td>
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<tr>
<td>N04-165</td>
<td>Environmental</td>
<td>2/29/04</td>
<td>Kuhio</td>
<td>Resistant</td>
<td>31-33</td>
</tr>
<tr>
<td>N04-166</td>
<td>Environmental</td>
<td>4/10/04</td>
<td>Ala Moana</td>
<td>Sensitive</td>
<td>36-10</td>
</tr>
</tbody>
</table>

D.O.C. = date of collection; LabID = experiment # and isolate # (Appendix D)

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Figure 6.1. Dendrogram of seawater *Staphylococcus aureus* isolates analyzed by pulsed-field gel electrophoresis at Hawaii Department of Health (isolate information in Table 6.5).
CHAPTER SEVEN

Study Recommendations and Future Studies

A. Recommendations

The following recommendations are based on the findings of this study.

1. The media CHROMagar™ Staph aureus (CSA) and CSA supplemented with 0.0035% sodium azide and 12g/L of glycine used with membrane filtration methods recommended for the recovery of Gram-positive bacteria as well as Staphylococcus aureus from marine recreational waters in Hawaii.

2. The use of CSA supplemented with 4µg/ml of oxacillin with membrane filtration and screening methods recommended to recover methicillin-resistant S. aureus from marine recreational waters in Hawaii.

3. Recommend the use of S. aureus and/or Gram-positive bacteria as microbial indicators for non-fecal pollution of marine bathing waters in Hawaii. Additional studies needed to set appropriate regulatory standards.

4. Physicians should be made aware of the risk of water-associated staphylococcal skin infections and should educate their patients on the possible transmission of this pathogen through recreational waters as well as person to person, or self infection, and that transmission is more probable in heavily used waters.

5. Posting of warning signs to inform beach goers with open cuts or sores of the infections hazards when they expose themselves to certain marine waters should be considered.
6. Bathers should be warned of the importance of adequate washing after exposure, especially where skin has been abraded or cut. Availability of freshwater for such cleaning purposes should be made available to the public, especially at heavily used beach areas.

B. Future Studies

Faster methods for the detection of *S. aureus* needed to ensure a timely response to pollution. Methods such as fluorescence in-situ hybridization (FISH), quantitative polymerase chain reaction (Q-PCR), or fluorescent staining followed by flow cytometry have been suggested. Indeed the use of Q-PCR has also been under investigation for use in monitoring for fecal indicators (*Enterococcus* spp.).

The recovery of stressed cells of *S. aureus* or methicillin-resistant *S. aureus* (MRSA) may be enhanced by the use of resuscitation methods similar to those listed in Standard Methods for the Examination of Water and Wastewater for enteric bacteria (Greenberg et al., 1992). In particular, the two-layer agar method where a thin layer of a general media (e.g. TSA) is poured over the selective media (e.g. CSA+O) may provide a more direct method of detecting MRSA than the methods employed in this study.

Another difficulty that was found in this study was the confirmation of resistance to oxacillin amongst the *S. aureus* isolates. The use of colony hybridization to identify MRSA directly on colony plates may provide a more feasible and accurate method to quantify levels of MRSA in seawater.

Identification of the non-target Gram-positive bacteria recovered from seawater on CSA would facilitate the finding of a more specific supplemental agent or selective conditions for improving inhibition of these background colonies.
The recovery and enumeration of staphylococcal bacteria from sewage samples needs further investigation. Appropriate media and dilutions of the samples for membrane filtration techniques will need assessment.

A 24-hour fluctuation study of staphylococcal counts at appropriate beaches should be conducted. Further study of the seasonal variation of *S. aureus* in marine waters needs to be done to clarify variances seen in this study. Physical factors of the natural waters which may affect bacterial activities such as current flow, wave height, tidal influences, and perhaps water temperature should be included in these assessments.

The investigation of *S. aureus* in marine recreational waters should be extended to other islands in Hawaii and other tropical areas where marine recreational activity are typically commonplace.

More direct associations of risk between bathing in polluted waters and subsequent transmission of *S. aureus* should be conducted. A prospective cohort type study has been considered, in which cultures from bathers prior to exposure and after exposure would be collected and compared in order to more directly show transmission of the pathogen amongst humans via seawater.

The behavior of *S. aureus* in marine waters and on sand needs further investigation. In particular, sand particles appear to enhance survival of *S. aureus* and biofilm formation on these particles should be considered. Biofilm formation is of particular interest with this pathogen as, in other species, there are reports of enhanced genetic exchange when in biofilm, which may implicate higher transmission of resistance genes.
CHAPTER EIGHT

Summary and Conclusions

The main components of this study included an evaluation of CHROMagar\textsuperscript{TM} Staphylococcus aureus (CSA) as a medium to directly recover and enumerate \textit{Staphylococcus aureus} from marine recreational waters, an examination to determine the prevalence of methicillin-resistant \textit{S. aureus} (MRSA) amongst the recovered isolates, and an investigation into association with clinical infections.

The first component focused on the assessment of media and methods for recovery and enumeration of \textit{S. aureus} using CSA. CSA was used to recover \textit{S. aureus} from marine and brackish waters and from sand samples. This medium was effective in recovering \textit{S. aureus} from marine waters but due to the interference of other bacterial growth, the identification of presumptive \textit{S. aureus} colonies was hindered in highly polluted waters. It was therefore concluded that CSA was effective in recovering the bacteria of interest from marine recreational waters, but that improvements to this medium were needed. Thus, an additional goal of the first component was to examine modifications of CSA to improve selectivity for \textit{S. aureus} from marine waters. Upon completing the assessment of several additives, the addition of 0.0035\% sodium azide to CSA was found to adequately improve selectivity without significantly affecting recovery. However, the addition of sodium azide significantly affected the morphology of the target colonies, which impeded adequate quantification and the addition of glycine improved this affect. Therefore, CSA modified with the addition of sodium azide (0.0035\%) and glycine (12 g/L; CSA+A+G) was useful in recovering \textit{S. aureus} due to its improved selectivity.
Following the evaluation of CSA and CSA+A+G, these media were used to survey the marine recreational waters around Oahu in order to determine the concentrations of this pathogen, and to examine the potential for *S. aureus* to multiply or survive in marine waters.

The final component of this study was to determine if there was some association between environmental *S. aureus* isolates recovered from marine recreational waters and clinically derived isolates using several strain-typing mechanisms to compare the two groups. In this study, the *S. aureus* isolates recovered from marine waters and clinical cultures were shown to have similar characteristics based on all of the typing methods used, which included antibiotic susceptibility patterns and ribotyping. In addition, typing of the marine isolates by pulsed-field gel electrophoresis and SCCmec determined that these isolates share similar genetic backgrounds to clinical *S. aureus* isolates recovered during outbreaks around the United States. Furthermore, several of the marine *S. aureus* isolates harbored important virulence genes, which indicate their potential to cause severe infections. Overall, these results implicate that, since similar strains can be recovered from both seawater and clinical specimens, marine recreational waters may serve as a source of transmission for virulent strains of *S. aureus* and MRSA.
Appendix A

Sampling data for selected marine water sites on Oahu with concentrations of people (approximately 250m$^2$) present at time of sampling and bacterial concentrations (CFU/100ml).

| Location     | Date      | Time  | EXP# | Total People | # bathers | # beachers | CSA (SA) | CSA (Total) | CSA+A (SA) | CSA+A (Total) | CSA+A+G (SA) | CSA+A+G (Total) |
|--------------|-----------|-------|------|--------------|-----------|------------|----------|-------------|------------|---------------|-------------|----------------|----------------|
| Magic Island | 7/20/03   | 16:00 | 8    | 143          | 97        | 46         | 38       | 458         | 11         | 102           |             |                |
| Magic Island | 8/3/03    | 14:30 | 10   | 111          | 76        | 35         | 25       | 540         | 12         | 125           |             |                |
| Ala Moana (1)| 8/16/03   | 16:00 | 12   | 114          | 77        | 37         | 30       | 680         | 22         | 129           |             |                |
| Ala Moana (1)| 9/6/03    | 14:00 | 14   | 174          | 122       | 52         | 58       | 1568        | 13         | 363           |             |                |
| Ala Moana (1)| 9/20/03   | 14:30 | 17   | 121          | 73        | 38         | 48       | 983         | 37         | 272           |             |                |
| Ala Moana (1)| 10/4/03   | 13:30 | 18   | 64           | 42        | 22         | 22       | 2040        | 16         | 225           |             |                |
| Ala Moana (1)| 11/1/03   | 14:45 | 23   | 89           |           |            | 16       | 1125        | 21         | 108           |             |                |
| Ala Moana (1)| 11/22/03  | 13:30 | 24   | 100          |           |            | 3        | 390         | 1          | 16            |             |                |
| Ala Moana (1)| 12/6/03   | 12:30 | 26   | 88           |           |            | 14       | 1380        | 1          | 58            |             |                |
| Ala Moana (1)| 4/10/04   | 12:25 | 36   | 125          |           |            | 10       | 280         | 0          | 95            |             |                |
| Ala Moana (1)| 6/6/04    | 13:35 | 42   | 100          | 60        | 40         | 3        | 1010        | 2          | 139           |             |                |
| Ala Moana (1)| 6/25/04   | 11:30 | 49   | 150          | 100       | 50         | 2        | 750         |             |               |             |                |
| Ala Moana (1)| 7/3/04    | 16:09 | 51   | 185          | 110       | 75         | 0        | 700         | 1          | >100          |             |                |
| Ala Moana (2)| 7/3/04    | 16:12 | 51   | 185          | 110       | 75         | 12       | 1960        |             |               |             |                |
### Appendix A (continued)

Sampling data for selected marine water sites on Oahu with concentrations of people (approximately 250m²) present at time of sampling and bacterial concentrations (CFU/100ml).

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### Appendix A (continued)

Sampling data for selected marine water sites on Oahu with concentrations of people (approximately 250m²) present at time of sampling and bacterial concentrations (CFU/100ml).

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Appendix A (continued)  Sampling data for selected marine water sites on Oahu with concentrations of people (approximately 250m²) present at time of sampling and bacterial concentrations (CFU/100ml).

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Appendix A (continued)  Sampling data for selected marine water sites on Oahu with concentrations of people (approximately 250m²) present at time of sampling and bacterial concentrations (CFU/100ml).

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<th># beachers</th>
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<th>CSA (Total)</th>
<th>CSA+A (SA)</th>
<th>CSA+A (Total)</th>
<th>CSA+A+G (SA)</th>
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EXP# = experiment number, SA = *Staphylococcus aureus*, TOTAL = total colony counts; CSA = CHROMagar<sup>TM</sup> Staph aureus, CSA+A = CSA with sodium azide, CSA+A+G = CSA+A with glycine
Bacterial concentrations (CFU/100ml) recovered from selected marine water sites on Oahu, including the number of isolates with some degree of methicillin resistant recovered using the indicated recovery media.

<table>
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<th>Time</th>
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<th>CSA+O (Total)</th>
<th># MRSA</th>
<th>MRSA media</th>
<th># MISA</th>
<th>MISA media</th>
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Bacterial concentrations (CFU/100ml) recovered from selected marine water sites on Oahu, including the number of isolates with some degree of methicillin resistant recovered using the indicated recovery media.

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<th>CSA+O (Total)</th>
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<th>MRSA media</th>
<th># MISA</th>
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</tbody>
</table>

EXP # = experiment number; Duke K = Duke Kahanamoku beach, number in parenthesis after site = particular location at the beach site; SA = Staphylococcus aureus. Total = total number of colonies; CSA = CHROMagar™ Staph aureus, CSA+O = CSA with oxacillin, CSA+10G = CSA with sodium azide and glycine, C+10SP = CSA with sodium pyruvate; TNTC = too numerous to count, strike through number = not confirmed as S. aureus
### Appendix C

Results from confirmation tests of presumptive *Staphylococcus aureus* and non-*S. aureus* isolates recovered from CHROMagar™ Staph aureus (CSA) containing media.

<table>
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<th>False+</th>
<th>Non-SA tested</th>
<th># confirmed</th>
<th>False-</th>
<th>SA tested</th>
<th># confirmed</th>
<th>False-</th>
<th>Non-SA tested</th>
<th># confirmed</th>
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Appendix C (continued) Results from confirmation tests of presumptive *Staphylococcus aureus* and non-*S. aureus* isolates recovered from CHROMagar™ Staph aureus (CSA) containing media.

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Appendix C (continued)  Results from confirmation tests of presumptive *Staphylococcus aureus* and non-*S. aureus* isolates recovered from CHROMagar™ Staph aureus (CSA) containing media.

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| TOTAL      | 27        | 25   | 2         | 59         | 45      | 14             | 18          | 8       | 10        | 109        | 103     | 6             |

Duke D = Duke Kahanamoku beach, number in parenthesis after name of location = specific area beach site, CSA+A = CSA with sodium azide, CSA+A+G = CSA+A with glycine, CSA+O = CSA with oxacillin, SA = *S. aureus*, False+ = mauve not confirmed as *S. aureus*, False- = non-mauve confirmed as *S. aureus*
## Appendix D

Confirmation and resistance tests for isolates recovered from marine waters on Oahu.

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**Confirmation and resistance tests for isolates recovered from marine waters on Oahu.**

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EXP#-experiment number; Cat-catalase test; Latex-Staphyloslide latex agglutination test; Probe-Gene Probe DNA:RNA hybridization test; OX-growth on CSA+O when used as a screening agar for recovered isolates; AM-Ala Moana; HB-Hanauma Bay; K-Kuhio; M.I.-Magic Island; S.S.-Sans Souci; W-Waimanalo; W.B. Waimea Bay; KASand-Kailua beach sand; pin.-pinpoint (<1mm); sm.-small (1-2mm); md.-medium (2-3mm); lg. - large (>3mm); drk.-dark; lt. - light; cntr.-center; [+/-] - positive; [-] - negative; [+/-] - weakly positive; [/-] - very weakly positive; S-susceptible; I-intermediate resistance; R-resistant; MIC-minimum inhibitory concentration; results in parenthesis are from re-test.
## Appendix E

Ribotypes and antibiotic susceptibility patterns of *Staphylococcus aureus* isolates from marine waters of Oahu.

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### Appendix E (continued)

Ribotypes and antibiotic susceptibility patterns of *Staphylococcus aureus* isolates from marine waters of Oahu.

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### Appendix E (continued)

Ribotypes and antibiotic susceptibility patterns of *Staphylococcus aureus* isolates from marine waters of Oahu.

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EXP#-experiment number; sim-similarity index; P-penicillin; GM-gentamicin; CTX-cefotaxime; TE-tetracycline; VA-vancomycin; E-erythromycin; CIP-ciprofloxacin; AM-ampicillin; R-resistant; I-Intermediate resistance; S-susceptible.
## Appendix F

Typing of environmental *Staphylococcus aureus* isolates by U.S. Center for Disease Control laboratories.

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### Appendix F (continued)  Typing of environmental *Staphylococcus aureus* isolates by U.S. Center for Disease Control laboratories.

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PFT = Pulsed-Field Type (McDougal et al., 2003); CDC = results from Centers for Disease Control; WA = PFT compared with Washington State Department of Health’s database; corresponding toxin gene acronyms are listed in Chapter 6 Materials and Methods section, Table 2; Toxin Genes: X = positive, blank = negative.
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