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# Performance of CHROMagar™ Staph aureus and CHROMagar™ MRSA for detection of *Staphylococcus aureus* in seawater and beach sand – Comparison of culture, agglutination, and molecular analyses

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ABSTRACT

Beach seawater and sand were analyzed for *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA) for samples collected from Avalon, and Doheny Beach, CA. Membrane filtration followed by incubation on CHROMagar™ Staph aureus (SCA) and CHROMagar™ MRSA (C-MRSA) was used to enumerate *S. aureus* and MRSA, respectively. Media performance was evaluated by comparing identification via colony morphology and latex agglutination tests to PCR (*clf*A, 16S, and *mec*A genes). Due to background color and crowding, picking colonies from membrane filters and streaking for isolation were sometimes necessary. The specificity of SCA and C-MRSA was improved if colony isolates were identified by the presence of a matte halo in addition to mauve color; however routine agglutination testing of isolates did not appear warranted. Using the appearance of a colony on the membrane filter in conjunction with isolate appearance, the positive % agreement, the negative % agreement, and the % positive predictive accuracy for SCA was 84%, 95%, and 99% respectively, and for C-MRSA it was 85%, 98%, and 92%, respectively. Sensitivity and specificity of SCA and C-MRSA with membrane-filtered beach samples were optimized through identification experience, control of filter volume and incubation time, and isolation of colonies needing further identification. With optimization, SCA and C-MRSA could be used for enumeration of *S. aureus* and MRSA from samples of beach water and sand. For the sites studied here, the frequency of detection of *S. aureus* ranged from 60 to 76% and 53 to 79% for samples of beach seawater and sand, respectively. The frequency of detection of MRSA ranged from 2 to 9% and 0 to 12% for samples of seawater and sand, respectively.

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*Abbreviations:* CHROMagar™ Staph aureus, (SCA); CHROMagar™ MRSA, (C-MRSA).

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## 1. Introduction

In an effort to protect human health, recreational waters are monitored for fecal indicating bacteria (e.g., enterococci, *Escherichia coli*) according to federal and state guidelines. One drawback to this approach is that these bacterial indicators do not address human pathogens that are not primarily associated with feces, such as *Staphylococcus aureus*.

*S. aureus* is a Gram positive bacterium primarily found on human skin and in nasal passages. Many people carry *S. aureus*, with estimates ranging from 20 to 40% of the population (Al-Zu'Bi et al., 2004; Kluytmans and Wertheim, 2005). *S. aureus* primarily is a commensal organism, but it is an opportunistic pathogen. Wound invasion, including self-inoculation can cause invasive infections resulting in a variety of diseases, including scalded skin syndrome, abscesses, septicemia, pneumonia, food poisoning, and toxic shock syndrome (Moore and Lindsay, 2001; Boyd and Brüssow, 2002).

There is concern that the prevalence of *S. aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) is on the rise in light of increased incidence of hospital-acquired, community-acquired, and community-onset infections throughout the world (Chambers, 2001; Zetola et al., 2005). Some of the community-acquired MRSA strains are particularly virulent (Baba, 2002). MRSA had been largely confined to hospitals and long-term care facilities, but it is now emerging in the community and occurring in healthy individuals with no traditional risk factors such as prior antibiotic exposure, admission to an intensive care unit, surgery, or exposure to MRSA-colonized patients (Chambers, 2001; Lema et al., 2005).

The skin is directly exposed to infectious agents during swimming (Henrickson et al., 2001), and a correlation between seawater exposure and *S. aureus* infection rates (Charoenca and Fujioka, 1995) suggests that recreational waters are a potential source of community-acquired *S. aureus* infections. It has long been recognized that *S. aureus* is released by swimmers (Robinton and Mood, 1966) who shed the bacterium from their nose, skin, and respiratory track (Charoenca and Fujioka, 1995). *S. aureus* is occasionally found in high abundance in recreational waters and sand, and the abundance is related to bather density and attributed to human activity (Charoenca and Fujioka, 1995; Papadakis et al., 1997; WHO, 2003). Shedding of $6 \times 10^6$ colony forming units (CFU) of *S. aureus* per person has been recorded in the first 15 min of bathing, and mass balance analysis supports the hypothesis that human shedding is a predominant source of *S. aureus* to coastal waters (Elmir et al., 2007). In addition to possible shedding from humans, animals are a potential source of *S. aureus*. For example, cases in which dogs acted as reservoirs for human re-infection by MRSA have been reported (Baptiste et al., 2009). Overall, staphylococci such as *S. aureus* have been suggested as an alternative or complementary indicator for marine water quality (Cheung et al., 1990; Gabutti et al., 2000).

The major obstacle to the use of *S. aureus* as an indicator of microbial pollution is the lack of a "recovery system that is sufficiently selective, differential, and reliable" for enumeration (Klapes, 1983). Currently, there are commercially available media for selective and differential growth of *S. aureus* and MRSA, such as CHROMagar™ Staph aureus (SCA) and CHROMagar™ MRSA (C-MRSA), respectively. These media have been evaluated using stock cultures and clinical isolates (Gaillot et al., 2000; Carricajo et al., 2001; Perry et al., 2004; Lema et al., 2005), but the performance with environmental samples has not been fully characterized. This study evaluated the performance of these media with membrane filtration of seawater and sand (extracted into PBS) in conjunction with colony isolation and latex agglutination testing and in comparison to molecular analyses.

## 2. Materials and methods

### 2.1. Sample collection

Samples of sand or seawater were collected during the Pacific Coast Water Study between July 26 and September 13, 2007 and between June 14 and August 24, 2008. In 2007 and 2008, samples were collected on Fridays, Saturdays, and Sundays from 3 sites (A, B, & C) at Avalon Beach, Catalina Island. In 2007, samples were collected at 8 AM, and in 2008 samples were collected at noon (a time of higher bather density). In 2008, samples also were collected from Descanso Beach, Catalina Island on Sundays at 8 AM. Historically, Descanso Beach demonstrated good water quality. In 2008, samples also were collected from Doheny Beach, CA on Saturdays at 8 AM from the mouth of San Juan Creek, enclosed behind a sand berm (site C) and approximately 100 yards away at an area of open-wave beach (site D) at both 8 AM and 1 PM.

### 2.2. Sample processing and enumeration

Sample collection was conducted by the Southern California Coastal Water Research Project (SCCWRP). Water was collected in 5 gallon buckets between ankle and knee depth on an incoming wave. All buckets, tanks, spigots, etc. were sanitized with 10% commercial bleach and dechlorinated with 1% sodium thiosulfate. Water was placed in 50 gallon tanks and stirred with 20 cm stir bars for 15 min before dispensing to ensure a well-mixed sample. Subsamples of seawater were placed into 1 gallon cubitainers and used for processing by membrane filtration. For bacterial culture analysis, aliquots of the subsample were filtered onto 47 mm, 0.45 μm, gridded mixed-cellulose esters membrane filters (EZ HAWG; Milipore, MA) and rinsed with approximately 20 ml phosphate buffered saline (PBS), according to standard membrane filtration protocols (Craig et al., 2002).

Sand was prepared for membrane filtration by vigorously hand shaking sand into PBS for 2 min using a ratio of 2 g sand to 80 ml of PBS (Baums et al., 2007; Goodwin et al., 2009). The solution of sand and dislodged particles was vacuum filtered through a sterile, 30 μm, 47 mm nylon net filter (Millipore, Bedford, MA, USA). An additional 10 ml rinse with PBS was used to remove any remaining sand from the shaking container. This procedure was repeated until a sufficient volume of "sandwater" was generated to satisfy the membrane filtration needs for that site. The "sandwater" was homogenized by hand mixing prior to filtration. Filters were incubated either on SCA or C-MRSA (BD Biosciences, San Jose,

CA, USA). SCA is a selective and differential medium for *S. aureus* and C-MRSA is a selective and differential medium for MRSA.

The volume of water filtered was adjusted throughout the study to yield the most readable plates. Typical filtration volumes for incubation on SCA were 80 ml and 150 ml, except for the Doheny Beach San Juan Creek site (site C), in which the typical volumes were 3 and 10 ml. The typical filtration volume for incubation on C-MRSA was 200 or 300 ml, except for Doheny Beach site C in which the typical volume was 50 ml. SCA filters typically were incubated at 37 °C for 24 h and C-MRSA filters were incubated at 37 °C for 48 h. Concentrations of *S. aureus* were calculated in terms of colony forming units (CFU) per 100 ml of water or CFU per gram dry sand. The water content of sand was determined by weighing sand aliquots before and after overnight drying at 110 °C.

Experiments were conducted to see if the addition of sodium azide (Fowler et al., 2004) and glycine (Fowler and Fujioka, personal communication) to SCA would help aid the ability to count colonies from environmental samples. A working stock of sodium azide and glycine (100 μl) was spread onto SCA plates for a final concentration of 0.0035% sodium azide and 12 g/L glycine, assuming adsorption into the top 1 ml of agar.

### 2.3. *Bacterial identification*

SCA is a selective and differential medium for *S. aureus* and C-MRSA is a selective and differential medium for MRSA. In both cases, target colonies are mauve colored (Table 1). Five colonies typically were picked from SCA membrane filters for each water and sand sample. Colony morphology was recorded and colonies were streaked for isolation (typically 2–3 times). Isolates with an appearance consistent with *S. aureus* (Table 1) were subjected to a latex agglutination test for *S. aureus* clumping factor and/or Protein A, as per manufacturer's instructions (BBL™ Staphyloslide™ Latex Test for *Staphylococcus aureus*; BD Biosciences, San Jose, CA, USA).

Many of the isolates were archived by scooping 5 large loopfuls of purified culture into Brain Heart Infusion Broth (BHI) with 15% glycerol and storing at −80 °C. Initially, colonies also were tested for catalase by placing colonies on a microscope slide, adding a drop of 3% hydrogen peroxide and looking for the generation of bubbles. A subset of colonies was analyzed by Gram Stain and by Vitek® analysis (bioMerieux, Durham, NC, USA).

DNA was obtained by placing an isolated colony in 50 μl of molecular biology grade water (MO BIO Laboratories, Carlsbad, CA) containing 1.25 μl of lysostaphin stock (Sigma #L7386 in MQ water; 2 mg/ml) for a final concentration of 50 μg/ml. The cell solution was vortexed for 10 s, heated to 95 °C for 10 min, and frozen overnight at −20 °C. The thawed extract was vortexed for 10 s and centrifuged to pellet cell debris. Strip tubes were centrifuged for 10 min in a picofuge and microfuge tubes were centrifuged for 1 min at 13,000×*g*. The supernatant was transferred to a clean, sterile tube and stored in a −20 °C chest freezer until used.

*S. aureus* identification was verified by polymerase chain reaction (PCR) amplification of the *clf*A gene (Mason et al., 2001) (Table 2). Colonies negative for the *clf*A gene were subjected to additional testing to verify that the reaction was a true negative. Colonies negative for *clf*A in singleplex PCR reactions were subjected to two multiplex reactions using the *clf*A and Staphylococcal 16S rRNA primer sets (Table 2). The first multiplex reaction contained DNA from the colony lysate and the second reaction contained the colony lysate DNA amended with an inhibition control (5 pg genomic *S. aureus* DNA, 0.1 pg/μl final concentration in MQ made from a 10 pg/μl stock of *S. aureus* subsp. *aureus*, methicillin resistant, ATCC 700699D-5).

Amplification reactions were carried out using 1X Phusion ™ HF Buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTPs (BioRad), 0.5 μM of each primer, 0.3 mg/ml bovine serum albumin (BSA), 0.5 μl (1 U) Finnzymes Phusion Hot Start High Fidelity DNA Polymerase (NEB, Ipswich, MA), 1 μl of cell colony lysate, and nuclease-free water for a final volume of 50 μl. Amplification conditions were as follows: 98 °C for 30 s; 35 cycles of 98 °C for 5 s, 60 °C for 10 s, 72 °C for 15 s; a final 8 min extension at 72 °C. Bands were visualized on 1 or 1.5% agarose gels run for 40 min at 120 V and compared to 6 μl of GelPilot 1 Kb Plus DNA ladder (Qiagen) or 2 μl of 100 bp-Low DNA ladder (Apex). Amplicon sizes are given in Table 2.

Each PCR run utilized two positive controls and one or two negative controls. The first positive control consisted of a reaction containing 10 pg of genomic *S. aureus* DNA. The second positive control consisted of a colony of *S. aureus*

**Table 1 – Parameters used to determine whether or not a bacterial colony grown on SCA or C-MRSA media was positive for *S. aureus*.**

| Test | Appearance | Conclusion |
|---|---|---|
| Colony on membrane filter | Mauve | Positive |
| | Non-mauve colony; colony with uneven edges, regardless of color | Negative |
| Colony streaked for isolation | Mauve w/matte halo | Positive |
| | Not mauve; colony with uneven edges, regardless of color; mauve without a matte halo | Negative |
| Latex agglutination | Positive, similar to control | Positive |
| | Absent, weak, or stringy agglutination | Negative |
| PCR, *clf*A gene | 638 bp band or band of 543 bp[a] size | Positive |
| | No band on agarose gel[b] | Negative |

a Colonies identified as *S. aureus* by Vitek and DNA sequencing, see text.
b After inhibition controls, see text.

**Table 2 – Information about primers used in this study (Mason et al., 2001).**

| Target gene | Primer name | Primer sequence 5′ → 3′ | Amplicon size (bp) |
|---|---|---|---|
| Staphylococcal 16S rRNA | 16S Staph-F | CCTATAAGACTGGGATAACTTCGGG | 791 |
| | 16S Staph-R | CTTTGAGTTTCAACCTTGCGGTCG | |
| *clfA* | *clfA*-F | GCAAAATCCAGCACAACAGGAAACGA | 638 |
| | *clfA*-R | CTTGATCTCCAGCCATAATTGGTGG | |
| *mecA* | *mecA* F | GCTTTGGTCTTTCTGCATTCCTGGA | 499 |
| | *mecA* R | GACACGATAGCCATCTTCATGTTGG | |

(ATCC #25923; Microbiologics) lysed by heat shock, as described above. Every run included a no template control in which molecular biology grade water (MO BIO Labs, Inc.) replaced the aliquot of DNA. Most runs included a second negative control prepared according to the procedure for colony lysis except that molecular biology grade water was used instead of a colony. Identification of MRSA was verified by PCR amplification of the *mecA* gene (Table 2) using the same PCR conditions as listed above.

A subset of colonies was used for partial sequencing of the 16S rRNA gene. The staphylococcal 16S rRNA gene primers (Table 2) were used for both PCR amplification and sequencing. PCR conditions were as described above. The sequence reaction was carried out according to the manufacturer's directions for the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and sequence analysis was performed on an ABI 3730 DNA Analyzer (Applied Biosystems).

### 2.4. *Data analysis*

Percent frequency of detection of *S. aureus* and MRSA were calculated as the percent of samples in which one or more *S. aureus* or MRSA colonies were detected compared to the total number of samples in which CFU data were successfully obtained. Data were analyzed for each sample matrix (seawater or sand) and beach (Doheny, Avalon). Avalon results were analyzed separately and combined by year (2007, 2008) (Table 3).

Media performance results were arranged in $2 \times 2$ contingency table format. In accordance with the nomenclature presented in those tables (Tables 4 and 5), performance statistics were calculated in terms of the positive % agreement ($100 \times a/(a+c)$) and the negative % agreement ($100 \times d/(b+d)$) between two tests. These values are equivalent to the % sensitivity and the % specificity if the reference test is the reference standard (FDA, 2007). The % positive predictive accuracy ($100 \times a/(a+b)$) also was calculated. This value corresponds to the probability that any particular positive test result is a true positive; assuming the comparison test is the reference standard and the data is representative of the population. Values were calculated using Simple Interactive Statistical Analysis (SISA) (http://www.quantitativeskills.com/sisa/statistics/diagnos.htm) and double checked using Clinical Calculator 1 (http://faculty.vassar.edu/lowry/VassarStats.html). The presented 95% confidence interval (CI) is the Wilson's CI, which is better than the normal approximation for circumstances in which the proportion is quite small or large, as is typical for diagnostic circumstances (Newcombe, 1998).

## 3. Results

Membrane-filtered seawater and sandwater were incubated directly onto C-MRSA (vs. initial growth on a more general medium and transferred to C-MRSA). The performance of SCA and C-MRSA was evaluated by picking putative *S. aureus* colonies from membrane filters used to process seawater and beach sand, plating for isolation on SCA or C-MRSA, evaluating the appearance of isolated colonies, subjecting isolated colonies to latex slide agglutination tests, and by amplification of genes to identify *S. aureus* or MRSA (Table 2). Initially, colonies also were tested for catalase, but all colonies tested were positive and this routine analysis was discontinued.

For SCA, the appearance of the original colony on the filter was recorded as positive or negative for *S. aureus* for 656 of the 1189 picked colonies. The appearance of isolated colonies was recorded for 1174 of the picked colonies, and agglutination tests were performed on 944 of the isolates. PCR amplification of the *clfA* gene was performed for 584 of the isolates, and 59% (346/584) of the colonies tested were *clfA* positive. This ratio should not be confused with the percent frequency of *S. aureus* detection in the samples themselves, which ranged from 60 to 76% for samples of seawater and 53 to 79% for samples of sand (Table 3).

**Table 3 – Percent frequency of *S. aureus* or MRSA detection in this study.**

| Colony type, sample matrix | Frequency of detection | | |
|---|---|---|---|
| | Doheny beach | Avalon 2007, 2008 | Avalon combined |
| *S. aureus*, seawater | 60% (9/21) | 73% (27/37); 76% (66/87) | 75% |
| *S. aureus*, sand | 53% (8/15) | 63% (40/64), 79% (70/89) | 72% |
| MRSA, seawater | 7% (1/15) | 2% (1/48); 9% (8/86) | 7% |
| MRSA, sand | 0% (0/21) | 2% (1/51); 12% (10/83) | 8% |

**Table 4 – Performance of SCA with samples of filtered seawater and beach sand in 2 × 2 contingency table format using PCR of the *clf*A gene as the comparison test.**

| Test result | Comparison test | | |
|---|---|---|---|
| | + | a | b |
| | − | c | d |
| | PCR clfA | | |
| | | + | − |
| Mauve colony on filter | + | 223 | 20 |
| | − | 21 | 37 |
| Mauve isolate with matte halo | + | 313 | 18 |
| | − | 33 | 220 |
| Latex agglutination | + | 301 | 25 |
| | − | 45 | 226 |
| Mauve isolate with matte halo and agglutination | + | 275 | 5 |
| | − | 71 | 226 |
| Mauve colony on filter, mauve isolate with matte halo and agglutination | + | 175 | 2 |
| | − | 69 | 55 |
| Mauve colony on filter, mauve isolate with matte halo | + | 206 | 3 |
| | − | 38 | 54 |

For C-MRSA plates, out of 310 picked colonies, the appearance of the colony on the filter was recorded for 215, the appearance of the isolate was recorded for 299 colonies, and agglutination tests were performed on 161 of the isolates. PCR amplification of the *clf*A gene was performed for 106 of the colonies isolated from C-MRSA, and 13% (14/106) were *clf*A positive, and thus determined to be *S. aureus*. Of the *clf*A positive colonies isolated on C-MRSA, 93% (13/14) were confirmed to be MRSA by PCR (both *clf*A and *mec*A positive); thus the overall ratio of MRSA isolated from C-MRSA was 12% (13/106). PCR amplification of the *mec*A gene was performed for 31 of the colonies, and 65% (20/31) had inherent antibiotic resistance as indicated by the presence of the *mec*A gene (Al-Zu'Bi et al., 2004). Of those positives, 65% (13/20) were *S. aureus* as confirmed by PCR of the *clf*A gene. These ratios should not be confused with the percent frequency of MRSA detection in the samples themselves, which ranged from 2 to 9% for samples of seawater and 0 to 12% in samples of sand, depending on the beach and the year sampled (Table 3).

### 3.1. *Utilization of matte halo as a criterion for identification of* S. aureus

According to the manufacturer's instructions, target colonies are mauve colored for SCA and C-MRSA. The ability of the media to specifically identify *S. aureus* isolates was improved if

**Table 5 – Performance of C-MRSA with samples of filtered seawater and beach sand in 2 × 2 contingency table format using PCR of the *clf*A gene as the comparison test.**

| Test result | Comparison test | | |
|---|---|---|---|
| | + | a | b |
| | − | c | d |
| | PCR *clf*A | | |
| | | + | − |
| Mauve colony on filter | + | 11 | 33 |
| | − | 2 | 27 |
| Mauve isolate with matte halo | + | 13 | 10 |
| | − | 0 | 83 |
| Latex agglutination | + | 12 | 8 |
| | − | 1 | 84 |
| Mauve isolate with matte halo and agglutination | + | 12 | 0 |
| | − | 1 | 92 |
| Mauve colony on filter, mauve isolate with matte halo and agglutination | + | 10 | 0 |
| | − | 3 | 59 |
| Mauve colony on filter, mauve isolate with matte halo | + | 11 | 1 |
| | − | 2 | 59 |

both mauve color and matte halo was used to classify an isolate as positive. Using the additional criteria of matte halo to identify *S. aureus* increased the % negative agreement from 76% (63/95) to 92% (95/95) for SCA. This increase in specificity was balanced by a decrease in sensitivity, but the % positive agreement remained at 90% for SCA (vs. 96% if color was the sole determinant); thus the combination of matte halo and mauve color was used for positive identification of *S. aureus* isolates for this study (Table 1).

### 3.2. Low molecular weight clfA bands

Out of the 690 total amplifications for the *clf*A gene (SCA and C-MRSA), 661 were of the expected 638 bp size (Table 2); however, 29 yielded a low molecular weight band of ~543 bp. Six of these 29 isolates were analyzed by Gram stain, Vitek® analysis (courtesy of SCCWRP), and partial DNA sequencing of the staphylococcal 16S rRNA gene (Table 2). All the isolates were identified as G(+) and as *S. aureus*. Based on these results, all 29 of the low molecular weight *clf*A amplifications were classified as *S. aureus* positive for use in 2 × 2 contingency tables (Tables 4 and 5). Interestingly, a band of this same molecular weight was observed for colonies isolated from a nasal swab taken from a human volunteer and the partial 16S rRNA sequence matched *S. aureus* and was identical to the sequences obtained from the environmental samples.

### 3.3. Evaluation of negative reactions

Multiplex PCR and inhibition controls were used in tandem to verify that colonies negative for the *clf*A gene were true negative reactions (Fig. 1). Negative reactions for the *clf*A gene were confirmed by the presence of a 16S band in the inhibition control. The presence of a 16S band in the sample lane indicated that although the colony was not *S. aureus*, it was a species of *Staphylococcus*. Inhibited reactions returned no or weak bands compared to the control. Inhibition was observed in only 17% (24/118) of the colonies tested, despite the fact that crude cell lysate was used as the template. Inhibited PCR reactions were diluted and re-amplified using the singleplex reaction.

The 16S/*clf*A reaction described in Mason et al. (2001) was used only to verify negative singleplex reactions because primer competition (Fig. 1) was observed for 37% of the colonies tested (53/91). The primer competition was evaluated further with amplification of lysed colonies of stock *Staphylococcus epidermidis* amended with varying amounts of *S. aureus* control DNA (0.02–10 pg). The *clf*A band was inhibited in all of these reactions, whereas both the *clf*A and 16S bands were observed for the reaction in which the *S. aureus* DNA was amplified alone (data not shown). Therefore, the multiplex reaction was deemed unsuitable for routine evaluation of environmental samples because a mixture of staphylococcal DNA with *S. aureus* DNA might cause a false-negative *clf*A reaction. Most of the *clf*A negative reactions that showed primer competition were double checked for PCR inhibition by running separate *clf*A and 16S reactions amended with inhibition control DNA, but there were no instances in which the *clf*A negative result was not confirmed (i.e., the presence of the 16S band in the multiplex confirmed a lack of PCR inhibition even in cases in which primer competition was observed).

### 3.4. Performance of SCA

The performance of SCA as a selective and differential medium for *S. aureus* with samples of seawater and beach sand was evaluated. In each contingency table, PCR of the *clf*A gene was used as the comparison test (Table 4) and performance was given in terms of the positive % agreement, negative % agreement, and the % predictive accuracy for a positive test (Table 6). SCA demonstrated the best balance of performance for determinations based on the appearance of isolated colonies (90% positive agreement, 92% negative agreement, 95% positive predictive accuracy). Overall, the % positive predictive accuracy of SCA was high (92–99%) for all of the parameters tested. The overall performance showed trade-offs between the positive % agreement and the negative % agreement. For example, although the positive % agreement was 91% when only colony appearance on a membrane filter was used, the negative % agreement was 65%. When the appearance on the filter was combined with the appearance of the isolate and agglutination testing, the positive % agreement dropped to 72%, but the negative % agreement increased to 96%. Interestingly, the positive % agreement improved to 84% and the negative % agreement remained high at 95% if

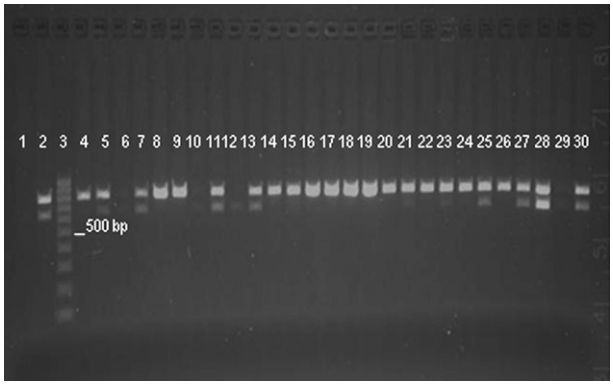


**Fig. 1 – Agarose gel electrophoresis of *clf*A/16S multiplex PCR reactions used to verify negative *clf*A results from singleplex reactions. Reactions confirmed negative for *clf*A and for staphylococcal 16S showed no bands for the samples (lanes 1, 6, 10, 12) and double bands for the corresponding inhibition controls (lanes 2, 7, 11, 13). Reactions confirmed negative for *clf*A but positively identified as *Staphylococcus* spp. showed 16S bands for the samples (lanes 4, 24, 26) and double bands for the inhibition controls (lanes 5, 25 and 27). Reactions that exhibited primer competition were confirmed *clf*A negative by 16S bands for both the samples (lanes 8, 14, 16, 18, 20, 22) and the inhibition controls (lanes 9, 15, 17, 19, 21, 23). Inhibited reactions (not shown) would have shown no bands in either the sample or inhibition control lanes. Control reactions consisted of a positive control for cell lysis (lane 28), a no template control (lane 29), and a positive control using stock *S. aureus* DNA (lane 30). Lane 3 contained a 100 bp DNA ladder.**

**Table 6 – Performance statistics[a] for SCA with samples of filtered seawater and beach sand using PCR of the *clfA* gene as the reference test.**

| Test on SCA: | Estimated value (95% CI lower limit, upper limit) | | |
|---|---|---|---|
| | Positive % agreement | Negative % agreement | % Positive predictive accuracy |
| Mauve colony on filter | 91 (87, 95) | 65 (51, 77) | 92 (87, 95) |
| Mauve isolate with matte halo | 90 (87, 93) | 92 (88,95) | 95 (91, 97) |
| Latex agglutination | 87 (83, 90) | 90 (86, 93) | 92 (89, 95) |
| Mauve isolate w/matte halo & agglutination | 86 (82, 90) | 98 (95, 99) | 98 (96, 99) |
| Mauve on filter, mauve isolate w/matte halo, & agglutination | 72 (66, 77) | 96 (87, 99) | 99 (96, 100) |
| Mauve on filter, mauve isolate w/matte halo | 84 (79, 89) | 95 (85, 99) | 99 (96, 100) |

a Calculated from Table 4, see Methods.

agglutination was not used to corroborate the identification of colonies from membrane filters (Table 6).

### 3.5. *Performance of C-MRSA*

The performance of C-MRSA as a selective and differential medium for MRSA when used with samples of seawater and beach sand is summarized in Tables 5 and 7. As with SCA, the *clfA* gene was used as the comparison test for C-MRSA. The *mecA* gene was not an appropriate reference test because that gene is present in a variety of staphylococci (Ubukata et al., 1990) and not only in *S. aureus*. The overall performance of C-MRSA increased markedly when *S. aureus* identification was based on the combination of appearance and agglutination results, with the % positive predictive accuracy increasing from 25% based solely on the appearance of filter colonies to 100% when a combination of colony appearance, isolate appearance, and agglutination testing was used. However, as seen with SCA, a more balanced performance was observed when agglutination testing was not included with the filter colony and isolate appearance (85% positive agreement, 98% negative agreement, 92% positive predictive accuracy; Table 7). The relatively small number of positive colonies may have contributed to the observed wide ranges in the 95% Wilson's Confidence Interval (e.g., 60–100% positive predictive accuracy for the combination of colony appearance on a filter and when isolated; Table 7).

### 3.6. *Amendments to reduce background*

It was at times difficult to categorize colony color on membrane filters because the filters themselves sometimes turned blue and/or pink and colonies sometimes touched, making color determination difficult or impossible. In an attempt to decrease the level of background, SCA plates were amended with sodium azide and glycine and results were compared to unaltered plates. Overall, adjustment of filter volume and incubation time (e.g., checking plates at 20 h to see if incubation should continue) seemed more helpful than amendment with sodium azide/glycine for regulating the amount of background color (data not shown), and sodium azide addition was not further pursued with these samples.

### 3.7. *Matrix effects*

The matrix of the sample used for membrane filtration (i.e., seawater or sand extracted into PBS) did not significantly affect performance of the media. For example, the

**Table 7 – Performance statistics[a] for C-MRSA with samples of filtered seawater and beach sand using PCR of the *clfA* gene as the reference test.**

| Test on C_MRSA: | Estimated value (95% CI lower limit, upper limit) | | |
|---|---|---|---|
| | Positive % agreement | Negative % agreement | % Positive predictive accuracy |
| Mauve colony on filter | 78 (49, 94) | 45 (32, 58) | 25 (14, 41) |
| Mauve isolate with matte halo | 100 (72, 99) | 89 (81,94) | 57 (35, 76) |
| Latex agglutination | 92 (62, 100) | 91 (83, 96) | 60 (36, 80) |
| Mauve isolate w/matte halo & agglutination | 92 (62, 100) | 100 (95, 100) | 100 (70, 100) |
| Mauve on filter, mauve isolate w/matte halo, & agglutination | 77 (46, 94) | 100 (92, 100) | 100 (66, 100) |
| Mauve on filter, mauve isolate w/matte halo | 85 (54, 97) | 98 (90, 100) | 92 (60, 100) |

a Calculated from Table 5, see Methods.

performance of SCA for seawater based on the appearance of a colony on the filter and when isolated was as follows: positive % agreement = 84% (111/131), 77–90% Wilson's CI; negative % agreement = 96% (25/26), 78–100% Wilson CI; % positive predictive accuracy = 99% (111/112), 94–100% Wilson CI. In comparison, the performance for sandwater was as follows: positive % agreement = 84% (95/113), 76–90% Wilson's CI; negative % agreement = 94% (29/31), 77–99% Wilson's CI; % positive predictive accuracy = 98% (95/97), 92–100% Wilson's CI. In addition, the frequency of detection of *S. aureus* and MRSA was similar for both seawater and sand samples (Table 3).

## 4. Discussion

Colony morphologies for stock cultures of *S. aureus* and *S. epidermidis* were consistent with the package inserts for SCA and C-MRSA. Matte halo was not mentioned as a determinant in the manufacture's literature, but it was mentioned by Gaillot et al. (2000) and Samra et al. (2004) as a possible help in diagnostics. This appears to be the first study that quantitatively defined the benefit of the matte halo criteria in terms of improved specificity (an increase from 76% to 92% positive agreement). It should be noted that although isolates could grow on SCA overnight, matte halo development sometimes required longer (~24–36 h) to develop.

Recovery experiments with *S. aureus* cultures were not performed here. However, recovery of more than 90% was reported for *S. aureus* stock strains plated on SCA from an inoculum of 10–100 CFU (Samra et al., 2004), and a recovery of 95% was reported for MRSA isolates on C-MRSA from clinical specimens (Flayhart et al., 2005). Particularly relevant to the work here, Fowler et al. (2004) reported ~75% recovery on SCA for *S. aureus* spiked into seawater and processed by membrane filtration. If the recovery reported by Fowler et al. (2004) is typical for environmental samples, the actual prevalence of *S. aureus* in seawater and sand would be higher than that indicated by the frequency of detection (Table 3).

Although estimates of sensitivity and specificity are often reported in the literature whether or not a reference standard is used, performance was presented here in terms of % agreement between two tests to avoid the assumption that PCR of the genes used in this study was the reference standard (FDA, 2007).

### 4.1. Comparison of SCA performance with other studies

The performance of SCA with seawater and sand samples was given in Table 6. In comparison, the reported sensitivity of SCA with clinical specimens ranged from 93.5 to 98.5% and specificity ranged from 97 to 100% (Gaillot et al., 2000; Carricajo et al., 2001; Samra et al., 2004). Carricajo et al. (2001) found that the addition of agglutination testing increased the specificity of SCA. That study used the Pastorex Staph Plus agglutination test, which was 75.5% specific. This study used the BBL Staphyloslide™ Test for *S. aureus* which has a reported specificity of 99% and a sensitivity of 100% (BD technical literature). The Staphyloslide™ agglutination test did increase the specificity of SCA for the colonies studied here. The addition of agglutination testing to the criteria of isolate appearance increased the negative % agreement from 92% to 98% for SCA (Table 6). More markedly, agglutination testing in conjunction with filter colony and isolate appearance increased the negative % agreement from 65% to 96% for C-MRSA. However, a balance of specificity and sensitivity was desired, and a better balance was achieved with membrane-filtered samples when agglutination testing was not included (Table 6). Therefore, the performance statistics did not justify the time and expense of agglutination testing for routine use.

The technical literature for the BBL Staphyloslide™ Test for *S. aureus* stated that "selective media such as Baird-Parker or mannitol salt agar may also give satisfactory results, but interpretation is more difficult due to the weak positive reactions and the tendency to produce stringy reactions." Compernolle et al. (2007) found that subjecting colonies taken directly from C-MRSA plates to Pastorex agglutination tests led to false-negative results. We used colonies directly from SCA plates and found stringy reactions in 13/45 and weak positive reactions in 18/45 of the *clf*A positive reactions that were considered agglutination negative (Table 1). These false-negative reactions are reflected in relatively poor positive % agreement values when agglutination was used in conjunction with appearance. However, stringy reactions were found in 61/226 and weak positive reactions in 42/226 of the agglutination negative/*clf*A negative reactions; therefore, reclassification of stringy and weak positive reactions as agglutination positive would not have been appropriate. Although growing colonies on another media prior to agglutination testing may have removed the false-negative results, the added effort and expense made this step untenable for routine classification of these environmental samples.

### 4.2. Comparison of C-MRSA performance with other studies

The performance of C-MRSA with seawater and sand samples at 48 h of incubation was given in Table 7. The performance of C-MRSA was relatively poor for colonies identified on membrane filters relative to identification of isolates. The addition of isolate appearance and agglutination testing made little change in the positive % agreement (78 vs. 77%), but it made a marked improvement in the negative % agreement (45 vs. 100%). In comparison, the reported sensitivity of C-MRSA with clinical specimens at 48 h ranged from 72 to 95.1% and the specificity ranged from 90 to 98.1% (Perry et al., 2004; Flayhart et al., 2005; Compernolle et al., 2007). Flayhart et al. (2005) reported performance statistics in comparison to *mec*A PCR, which they accepted as the "gold standard" for identification. In that case, sensitivity and specificity were 95.1% and 98.1%, respectively. In this study, a combination of *clf*A and *mec*A PCR was used, returning positive and negative % agreement values of 100% and 89%, respectively for the appearance of isolates on C-MRSA.

Compernolle et al. (2007) reported that the addition of agglutination testing increased the specificity of C-MRSA with clinical samples from 90 to 95.8% after 48 h, and that the addition of Gram staining offered no further gains in performance. We found the addition of agglutination testing to isolate appearance increased the specificity from 89 to 100% (Table 7). However, analogous to the results with SCA, the

overall performance statistics did not justify the costs of routine agglutination testing because a better balance between positive and negative % agreement was achieved when agglutination data was not considered (Table 7).

### 4.3. *Statistical limitations*

Accurate estimates of sensitivity and specificity assume that the experiment tested both true positive and true negative colonies. In contrast, this study was biased against negative colonies because it focused on putative *S. aureus* colonies. From a statistical perspective, this translates to an overestimation of the prevalence of *S. aureus* in the population, with consequences to the performance statistics (FDA, 2007). Therefore, the % positive predictive accuracy ($100 \times a/(a+b)$) also was presented because this value was not expected to be affected by a bias against negative colonies. One consequence of a bias against negative colonies is that the values presented here should represent a worst case scenario for the negative % agreement (aka % specificity when the reference standard is used).

### 4.4. *Differences between clinical and environmental studies*

A major difference between clinical evaluations of CSA and C-MRSA and the study performed here is that colonies first had to be identified on a membrane filter. For isolates, the matte halo criteria increased the specificity of SCA and C-MRSA, but this aid could not be utilized for colonies on a membrane filter. Furthermore, interpretation of colony color on the membrane filter was sometimes not straight forward, particularly for aliquots of seawater incubated on SCA. The filter could be a mixture of blue and pink hues, and it was not uncommon for colonies of different colors to be touching or on top of one another. Such circumstances made classification of colony color difficult or impossible and required colony isolation and testing. Adjustment of incubation times and the volume of water filtered helped control background color and maintain independent colony growth. Amendment of plates with sodium azide (Fowler et al., 2004) and glycine did not appear to be of significant aid for the samples tested here.

A difference in the application of SCA and C-MRSA for clinical studies and for the application studied here (use with environmental samples) is that clinical studies primarily looked for (+)/(−) detection from a sample. In contrast, a desired application for SCA and C-MRSA is enumeration of *S. aureus* and MRSA, particularly for use in epidemiology studies, and this places more stringent requirements on the media. Environmental samples present a particular challenge because even those samples that maintained a white filter and isolated colonies often would present colonies of various shades of pink. We found that technician experience improved the proper identification of *S. aureus* colonies on membrane filters and for isolates. It appears that with an experienced technician, control of filter volumes, control of incubation times, and with isolation of colonies needing further identification, SCA and C-MRSA can be used for enumeration of *S. aureus* and MRSA from samples of seawater and beach sand.

## 5. Conclusions

- SCA and C-MRSA can provide a good balance of sensitivity and specificity for analysis of *S. aureus* and MRSA in seawater and beach sand when membrane filtration and incubation conditions are optimized and when used in conjunction with the appearance of colonies isolated from filters.
- For isolated colonies, utilization of a matte halo criteria in addition to mauve coloration improved specificity. This appears to be the first instance in which utilization of the matte halo has been quantitatively shown to improve media performance. Agglutination testing of isolates increased the negative % agreement, but routine use was not justified because better overall performance (sensitivity balanced by specificity) was achieved when agglutination results were not considered.
- For samples collected from Avalon (2007, 2008) and Doheny (2008) beaches, the frequency of detection for *S. aureus* (seawater 60–76%; sand 53–79%) was higher than for MRSA (seawater 2–9%; sand 0–12%). Few colonies isolated from C-MRSA were verified to be MRSA via positive detection of both the *clf*A and *mec*A genes; however, inherent antibiotic resistance, as indicated by just detection of the *mec*A gene, was more prevalent.

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